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MOLECULAR INTERACTIONS OF  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$   
WITH OUABAIN AND THE MEMBRANE

by



ANTHONY FELIX ALMEIDA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH  
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THE UNIVERSITY OF ALBERTA  
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Molecular interactions of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  with ouabain and the membrane", submitted by Anthony F. Almeida in partial fulfilment of the requirements for the degree of Doctor of Philosophy.





For my daughters Elise, Camille and Diana who were  
all too young to be aware of their contribution.



## ABSTRACT

The membrane bound enzyme ( $\text{Na}^+ + \text{K}^+$ )-ATPase has a characteristic sensitivity to temperature such that it is more efficient above a critical temperature ( $T_c$ ) than below it. This phenomenon is frequently displayed as an Arrhenius plot ( $\log \text{ rate } v/s \ 1/T$ ) from which two activation energies  $E_{a1}$  and  $E_{a2}$  can be derived. This temperature dependence is believed to involve fluidity changes in the membrane lipids.

Lipid modification of ( $\text{Na}^+ + \text{K}^+$ )-ATPase enriched membranes from rabbit kidney and from ox brain, was achieved by treatment with deoxycholic acid (DOC), with sodium dodecyl sulfate (SDS), or by lipolysis with phospholipase-A. Changes in the membrane consequent to treatment were examined in three ways:

- a) The effects of temperature on the specific activity of the enzyme.
- b) The effects of temperature on the binding of [ $^3\text{H}$ ]-ouabain to the enzyme.
- c) The changes in membrane fluidity using electron spin resonance (ESR).

The following observations were made:

### 1. Specific activity of ( $\text{Na}^+ + \text{K}^+$ )-ATPase

Detergent treatment increased the specific activity of the enzyme, but the non-linearity of the Arrhenius plot remained.  $E_{a1}$  and  $E_{a2}$  varied slightly, but maintained a constant relationship ( $E_{a1}:E_{a2}$ ) of about 0.5.

PPL-A caused an initial increase in specific activity of untreated membranes followed by a progressive decline. Detergent





treated membranes showed only a progressive decline in activity.

## 2. Ouabain binding to $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$

As the enzyme was purified by detergent treatment, the affinity of the drug for the receptor was reduced. Equilibrium binding was also reduced but to a lesser degree.

Temperature affected the rate but not the equilibrium binding of the drug. Binding rates, when presented in the Arrhenius form yielded a non-linear plot for untreated membranes, but not for detergent treated or PPL-A treated membranes.

Drug binding sites and catalytic sites increased together as the enzyme was purified. In contrast, PPL-A caused a greater loss of catalytic sites than of drug binding sites.

## 3. ESR studies with the lipid spin probe M 12-NSE

$(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enriched membranes exhibit two binding sites for M 12-NSE which permit unrestricted or restricted motion. These two populations, designated U and R sites respectively, have been characterized. R sites are in the core of the bilayer; the location of U sites is uncertain.

Changes in probe mobility with temperature differ for U and R sites. In the Arrhenius form, mobility changes at U sites are linear while at R sites are non-linear. M 12-NSE at R sites experiences three distinct events at 30°C, 20°C and 10°C, which may be phase transitions and separations; after DOC treatment, two events at about 25°C and 10°C were observed, while after SDS treatment only a single discontinuity at about 18°C was obtained. These differences were not apparent in liposomes made from total lipid extracts of treated and untreated membranes.



ESR studies of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  also provided evidence for changes in the width of the biomembrane on cooling; in contrast, liposomes made from lipid extracts of these membranes were markedly different.

The following conclusions are drawn:

1. Lipids associated with the drug binding site differ in quality and function from those associated with the catalytic activity.
2. Purification results in an increased homogeneity of lipids associated with the enzyme.
3. The enzyme exhibits intimate protein-lipid interactions.
4. It is possible that the energy transduction mechanism of the sodium-pump incorporates the potential energy of an elastic membrane.





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I thank my supervisor Dr. John S. Charnock. He found the patience and understanding to be teacher and friend. He always listened with attention and enthusiasm to any suggestions and ideas, no matter how weak or flawed the logic. "Let me think about it" he would say and give me time to find the flaws myself. "John Charnock, I acknowledge this masterful way of protecting my pride and preserving my originality." I will not forget this lesson.

I doff my cap in the direction of the Chemistry Department and remember that I used their ESR spectrometer; and while in this position of respectfulness, I salute Dr. R.E.D. McClung who began at the very beginning and taught me the essentials of electron spin resonance spectroscopy.

Many others have helped in one way or another and I thank them collectively; but Laurie Simonson I thank singularly.

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## INTRODUCTION



## General

Interest in the cardiac glycosides, a group of drugs of which digitalis is the most widely known, developed along two lines, namely the clinical and the biochemical. Withering is generally credited with having introduced digitalis to the practice of medicine in 1785, but it was not until 1953 that Schatzmann reported the presence of a cation pump in red blood cells that could be inhibited by ouabain. The pump was later shown to be the sodium-plus-potassium-activated-adenosine triphosphatase ( $\text{Na}^+ + \text{K}^+$ )-ATPase. According to Withering (1783) digitalis was a drug with very wide application but in 1940, Gold and Cattell showed the inotropic effect to be a consequence of the direct action of digitalis on the myocardium. The three areas, namely the drugs, the clinical pharmacology, and the biochemical pharmacology will be discussed separately in the introduction to this dissertation, but this subdivision does not imply that these areas are unrelated.

## Drugs

The cardiac glycosides are composed of an aglycone or genin and a sugar. The sugar moiety may vary in number from one to four molecules, and also in type. Although pharmacological activity is confined to the aglycone, the sugars may influence water solubility and drug penetrability into cells. The suggestion has also been made that the sugar may occupy a subsite on the receptor for these drugs (Erdmann and Schoner, 1974). A disaccharide increases the affinity of the drug, but a trisaccharide decreases it; thus, some structural specificity of the molecule may also exist for the receptor subsite. Qualitatively the different cardiac glycosides are similar in their pharmacological effects.



*In vitro* experiments generally utilize ouabain as it exhibits greater aqueous solubility.

The pharmacological similarity of these drugs is also manifested in their chemical structure, which is basically a steroid nucleus with the glycoside linkage at the C<sub>3</sub>-OH position and an unsaturated five or six membered lactone ring at C<sub>17</sub>. Saturation of the lactone ring reduces the activity by an order of magnitude while opening of the ring abolishes the activity altogether. A secondary OH at C<sub>3</sub> and an OH at C<sub>14</sub> are believed to be essential components of stereochemical configuration. The cis A:B isomer is also an essential structural requirement in that the trans A:B isomer suffers a fifty-fold loss of binding (Erdmann and Schoner, 1974).

### Clinical Pharmacology

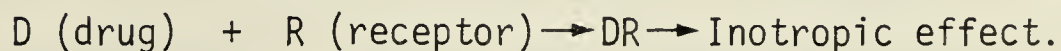
The clinical effects of the cardiac glycosides whether beneficial or toxic are qualitatively similar. The drugs affect the mechanical as well as the electrical properties of the heart. When used in the patient with a congested heart, the beneficial effects are a consequence of the improved force of contraction of the ventricle, that is, the positive inotropic effect. It is not yet clear whether the inotropic response and the toxic effects are mediated through distinct receptors. Indeed the contrary might be true, with the two effects reflecting a difference only in magnitude. The relationships between lethal doses of the drugs on the one hand and I<sub>50</sub> for the inhibition of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase on the other was observed by Repke in 1963, and it led him to suggest that the enzyme might be the digitalis receptor.

However the precise role of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase in the inotropic effect of digitalis on the heart remains a mystery. Essentially





most investigators (Schwartz, 1976; Aker, 1977) have approached the problem from the beginning or the end of the following reaction sequence:



The studies on drug receptor interactions have sought to establish a correlation between binding of the drug to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  and its inotropic effect on the heart. These investigations have included whole animal experiments as well as isolated hearts and strips of cardiac tissue. The following is a brief summary of the findings:

- (1) Only cardiotonic steroids display enzyme inhibition (Repke, 1961; Matsui and Schwartz, 1967; Matsui and Schwartz, 1968; Albers *et al.*, 1968).
- (2) By using experiments that combine *in vivo* and *in vitro* methods, the development of the inotropic response was shown to correlate with drug binding to the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  (Besch *et al.*, 1970).
- (3) Also from combination experiments of the type described above, the observation was made that the drug either did not bind to or was easily dissociated from mitochondria and from sarcoplasmic reticulum (Allen *et al.*, 1975; Jones *et al.*, 1976).
- (4) The cardiac glycosides exhibit a range of sensitivities. Beef, human, and dog hearts are at the sensitive end and rats on the insensitive end of the scale (Detweiler, 1967). Rabbits occupy an intermediate position. Inhibition of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  preparations from different species shows a correlation between the sensitivity of the species and the  $I_{50}$  which was time dependent. In the case of the rat the  $I_{50}$  was both lower and not time dependent (Aker *et al.*, 1969; Albers *et al.*, 1968; Allen and





Schwartz, 1969; Erdmann and Schoner, 1973a; Schwartz *et al.*, 1969).

- (5) The affinity of [ $^3\text{H}$ ]-ouabain binding to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  preparations from different animals showed correspondence with their drug sensitivity. That is, high sensitivity coincided with high affinity of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  for the drug (Repke, 1961; Albers *et al.*, 1968; Schoner *et al.*, 1972).
- (6) On the species sensitivity scale, dissociation of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase-drug}$  complex correlated positively with reversal of the pharmacological effect on isolated hearts as achieved by washing out the drug (Akeris *et al.*, 1972).
- (7) Some reports contradict the correlations discussed above (Okita, 1973; Okita *et al.*, 1973; Roth-Schechter *et al.*, 1970; Dutta and Marks, 1969). They are however considerably weakened by the following: (a) the test animal was the rabbit, which occupies an intermediate position on the species-sensitivity series (Dutta and Marks, 1969; Okita, 1973; Okita *et al.*, 1973), (b) the low  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity of the control membrane preparations (Dutta and Marks, 1969; Roth-Schechter *et al.*, 1970; Okita, 1973; Okita *et al.*, 1973) and (c) use of the drug strophanthidin-3-bromoacetate which differs from digitalis in its binding specificity (Dutta and Marks, 1969; Hokin, 1969; Fricke and Klaus, 1971).

Thus, the information from drug-receptor interactions favour some manner of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  involvement in the inotropic action of these drugs.

At the other end of the sequence (p. 4), the workers have begun with the very reasonable premise that the increased force of contraction resulting from treatment with these drugs, proclaims an altered



calcium pool(s). It is an established fact that the calcium ( $\text{Ca}^{++}$ ) in beating hearts is increased after treatment with cardiac glycosides (Gold and Cattell, 1940; Lee and Klaus, 1971; Marks and Weissler, 1972; Smith, 1973; Langer, 1974), but further characterisation of this phenomenon has not been attained. It is unlikely that digitalis affects  $\text{Ca}^{++}$  pools associated with the mitochondria or the contractile proteins (Lee and Klaus, 1971).

It is known that  $\text{Ca}^{++}$  inhibits the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  in the presence of sodium ions ( $\text{Na}^+$ ) and potassium ions ( $\text{K}^+$ ) (Skou, 1957, Skou, 1965), but in the absence of  $\text{Na}^+$ , or with low  $\text{Na}^+$ , the enzyme may be activated (Schon *et al.*, 1972). Finally the suggestion has been made that the affinity of  $\text{Ca}^{++}$  for some phospholipid in the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  preparation may be altered by ouabain (Gervais *et al.*, 1975).

The information from studies of  $\text{Ca}^{++}$  involvement in the inotropic effects suggests a number of possible mechanisms. Three of these postulates have been presented by Schwartz (1976) in a recent review and are listed below:

- a) Inhibition of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , leads to increased intracellular  $\text{Na}^+$  and consequent increase of intracellular  $\text{Ca}^{++}$  (Baker, 1972; Langer, 1973; Reuter, 1974).
- b) Inhibition of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  by cardiac glycosides leads to altered  $\text{Ca}^{++}$ -affinity inside the cell.
- c) Inhibition of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  may lead to loss of  $\text{K}^+$  causing a  $\text{K}^+$  directed influx of  $\text{Ca}^{++}$ .

Nevertheless it must be emphasized that postulates are as yet speculative and the final statement must remain that the specific binding of  $\text{Ca}^{++}$  to the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  during the inotropic effect has not been demon-



strated (Schwartz, 1976).

## Biochemical Pharmacology

### 1. General

The majority of animal cells possess a high intracellular concentration of  $K^+$  and a low concentration of  $Na^+$ . To maintain this state, a so-called "coupled pump" mechanism is necessary, so that both  $K^+$  and  $Na^+$  can be moved simultaneously against their concentration gradients but in opposite directions. After the discovery of a cation pump in red blood cells by Schatzmann (1953), there was a tremendous drive to locate an enzyme that would fill the role of the pump. In 1957 Skou described such an enzyme in fragmented nerve membranes from the walking legs of crabs. This enzyme required  $Na^+$  and  $K^+$  for full activation and utilized adenosine triphosphate (ATP) as its substrate. It was found to be a sodium-plus-potassium-dependent magnesium ATPase now known as  $(Na^+ + K^+)$ -ATPase. Thus two associated phenomena can be defined, namely the derivation of chemical energy from ATP by the enzyme and its utilization by the pump for physical work. The similarities of the two processes are now well recognized, and have been extensively reviewed (Wheeler and Whittam, 1970; Skou, 1971) particularly in an article by Glynn (1968). Some of the similarities between the pump and the enzyme are as follows:

- i. The pumping of  $Na^+$  from within the red cell occurs only if  $K^+$  is present on the outside of the cell. This simultaneous presence of  $Na^+$  and  $K^+$  is also a requirement for the  $(Na^+ + K^+)$ -ATPase activity of broken membrane preparations.

- ii. Inhibition of  $Na^+$  and  $K^+$  pumping on the one hand and  $(Na^+ + K^+)$ -





ATPase activity on the other, occur at similar concentrations of the cardiotonic glycosides.

iii. The structure-activity relationships of the cardiac glycoside molecules are common to both transport inhibition as well as  $(\text{Na}^+ + \text{K}^+)$ -ATPase inhibition.

iv.  $\text{K}^+$  and ouabain compete in both mechanisms.

v. The optimal concentration of  $\text{K}^+$  and of  $\text{Na}^+$  is the same for both processes.

vi. Both processes display vectorial qualities, with a requirement for  $\text{Na}^+$  and ATP inside the cell and  $\text{K}^+$  and ouabain outside the cell.

This evidence clearly shows that the transport mechanism and the  $(\text{Na}^+ + \text{K}^+)$ -ATPase activity are closely related phenomena. Thus it is currently accepted that the transport of  $\text{Na}^+$  and  $\text{K}^+$  in many cells is a process that derives energy directly from the hydrolysis of ATP via  $(\text{Na}^+ + \text{K}^+)$ -ATPase.

## 2. Catalytic aspects

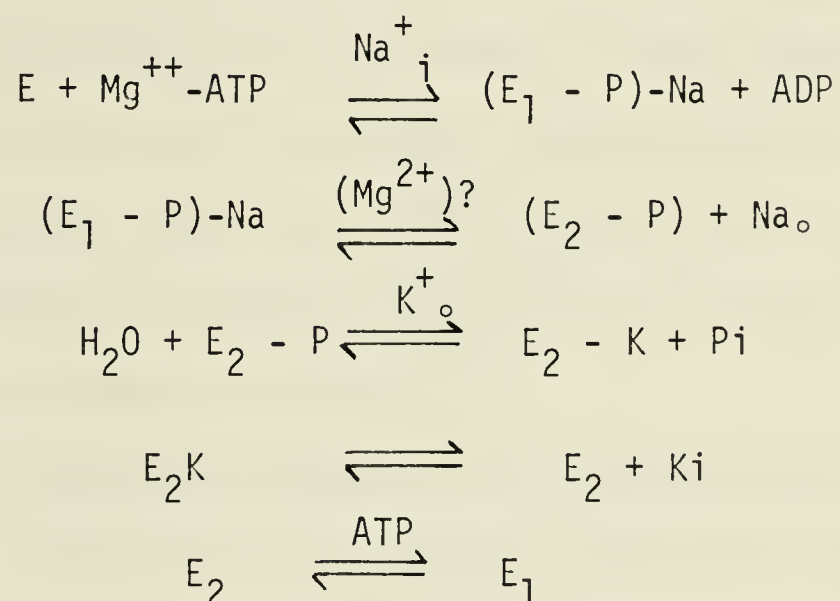
For full activation this enzyme requires magnesium ions ( $\text{Mg}^{++}$ ),  $\text{Na}^+$ , and  $\text{K}^+$ , and is inhibited by the cardiac glycosides. In the absence of  $\text{Na}^+$  and  $\text{K}^+$ , membrane hydrolysis of ATP still occurs, but at a greatly reduced rate. This component, which requires  $\text{Mg}^{++}$  but not  $\text{Na}^+$  or  $\text{K}^+$  for activation is variously referred to as  $\text{Mg}^{++}$ -ATPase, ouabain insensitive ATPase or basal ATPase. In this dissertation, the term  $(\text{Na}^+ + \text{K}^+)$ -ATPase will be used to indicate that component of membrane ATP hydrolysis which depends on  $\text{Mg}^{++}$ ,  $\text{Na}^+$ , and  $\text{K}^+$ , and which is completely inhibited by ouabain.

The hydrolysis of ATP by the  $(\text{Na}^+ + \text{K}^+)$ -ATPase is a complex process and thus presents a number of facets for investigation. Thus,





the substrate specificity, the characteristics of the cations and the inhibition by ouabain have all been examined extensively. This has generated a vast amount of information, but a complete understanding has not yet been achieved. The following reaction mechanism, although incomplete, is reproduced from Schwartz (1976) to facilitate discussion of the different facets of the problem.



ATP is the most effective of the nucleoside triphosphates (Charnock *et al.*, 1963), with CTP (Hokin and Yoda, 1964; Matsui and Schwartz, 1966; Towle and Copenhaver, 1970) and ITP (Matsui and Schwartz, 1966; Towle and Copenhaver, 1970) being less effective and GTP (Matsui and Schwartz, 1966; Schoner *et al.*, 1968) only marginally so. The enzyme does not display ouabain-sensitive hydrolysis of UTP (Hokin and Yoda, 1964). The 6-amino group of the purine ring and the  $\gamma$ -phosphate are deemed essential for tight binding and  $Mg^{++}$ -ATP appears to be the true substrate for this enzyme (Hexum *et al.*, 1970), although work by Hegyvary and Post (1971) casts some doubt on the latter statement. There is ample evidence to show that the enzyme-nucleoside bond is an acylphosphate (Nagano *et al.*, 1965; Hokin *et al.*, 1965; Alexander and Rodnight, 1970), which has now been characterized as  $\beta$ -aspartylphosphate



(Post and Orcutt, 1973; Post and Kume, 1973; Hokin, 1974; Degani *et al.*, 1974).

The cation requirements of this enzyme have been studied extensively. Hexum *et al.* (1970) have shown from studies of catalytic rates that  $Mg^{++}$ -ATP is the substrate for this enzyme, and free ATP or free  $Mg^{++}$  results in competitive and noncompetitive antagonism. Thus, not only is  $Mg^{++}$  essential for the enzyme, it must also be maintained at a concentration about equal to the ATP. Other divalent cations, namely manganese and cobalt can replace  $Mg^{++}$  but the over all efficiency of the hydrolytic reaction is reduced by 90% (Sachs and Welt, 1967; Atkinson *et al.*, 1968; Atkinson and Lowe, 1972).

The interaction between the sodium pump and the two transported cations  $Na^+$  and  $K^+$  can be discussed under five different categories or sub-headings as was recently described by Glynn and Karlsh (1975).

1.  $Na^+$ - $K^+$  exchange
2. Reversed  $Na^+$ - $K^+$  exchange
3.  $Na^+$ - $Na^+$  exchange
4.  $K^+$ - $K^+$  exchange, and
5.  $Na^+$  efflux without exchange.

The first of these five modes, the  $Na^+$ - $K^+$  exchange is the normal or forward mode of operation of the sodium pump, while the remainder are achieved only by manipulation of the operating conditions. Although the investigations of these so called partial reactions of the pump (Glynn *et al.*, 1971) have contributed valuable information, they are not considered pertinent to this dissertation and will not be discussed.



Under normal physiological conditions the sodium pump in a variety of tissues including human red blood cells, nerve and muscle, display a similar stoichiometry (Bonting and Caravaggio, 1963; Sen and Post, 1964; Baker, 1965; Whittam and Agar, 1965; Garrahan and Glynn, 1967; Dudding and Winter, 1971; Garay and Garrahan, 1973; Ellory and Carleton, 1974). For every molecule of ATP that is hydrolyzed, three  $\text{Na}^+$  are pumped out in exchange for two  $\text{K}^+$ . The sodium pump would therefore appear to be electrogenic when operating in its normal mode. However, the suggestion has been made that a proton from water may move inward with the potassium ion thereby making the pump electrically neutral (Schwartz *et al.*, 1975).

The studies undertaken to investigate interactions between cations and the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  are mostly kinetic in nature, and employ enzyme enriched membrane fragments. Although often vesicular in form, these membrane fragments do not maintain ionic gradients. Therefore, extrapolation of the information to a sodium pump in an intact cell may not be valid. It is generally accepted that there are three sodium sites on the inside and two potassium sites on the outside of the cell membrane. Using a brain membrane preparation, Schwartz' group have shown that the apparent affinity of the sodium sites but not the potassium sites is equivalent (Lindenmayer *et al.*, 1974). Different affinities for potassium were also reported for erythrocytes by Garahan and Glynn (1967) and by Ellory and Carleton (1974).

The  $\text{Na}^+:\text{K}^+$  ratio is optimal over a range between 5:1 and 10:1. For normal function the pump has an absolute requirement for sodium (Dahl and Hokin, 1974), but a variety of cations will substitute for potassium (Britten and Blank, 1968; Skulskii and Manninen, 1973), to







achieve different degrees of efficiency.

### 3. Ouabain binding studies

The reaction sequence shown previously (page 8) depicts two phosphorylated intermediates. Considerable evidence exists to support this concept and the transformation of the  $E_1$ -P state to a lower energy  $E_2$ -P state is currently well accepted (Charnock *et al.*, 1963; Rose, 1963; Charnock and Post, 1963; Albers *et al.*, 1963; Hokin *et al.*, 1965; Kanazawa *et al.*, 1967).  $Mg^{++}$  as well as  $Na^+$  are required for this conversion and in the presence of potassium ions the  $E_2$ -P complex is dephosphorylated (Post *et al.*, 1965). It is also generally accepted that the two states represent two conformations of the phosphorylated enzyme (Post *et al.*, 1969; Post *et al.*, 1972). Despite its general acceptance, the role of this conformation change in the translocation of  $Na^+$  and  $K^+$  has not been explained in any detailed molecular way.

In 1968 it was reported that the  $(Na^+ + K^+)$ -ATPase could be phosphorylated in the presence of inorganic phosphate (Pi) and  $Mg^{++}$  (Albers *et al.*, 1968; Lindenmeyer *et al.*, 1968). Sodium inhibited the formation of this particular E-Pi complex but  $K^+$  had no effect (Lindenmeyer *et al.*, 1968), suggesting that E-Pi is not identical to the  $E_2$ -P complex referred to above. Work from a number of different laboratories, applying a variety of methods, supports the concept that the  $E_2$ -P differs from the E-Pi (Lindenmeyer *et al.*, 1968; Hart and Titus, 1973a; Hart and Titus, 1973b; Akera *et al.*, 1974; Schwartz *et al.*, 1974).

The binding of ouabain to both complexes has been extensively studied, although inhibition of the enzyme activity necessitates binding to  $E_2$ -P. Two underlying questions have prompted workers to seek a



better understanding of the phospho-enzyme complexes:

- i) Will information on the ouabain-enzyme interaction reveal molecular characteristics of the sodium pump?
- ii) Will the pharmacological actions of these drugs, namely the therapeutic and the toxic effects, be better understood by examining the binding of ouabain to the enzyme?

The interaction of cardiac glycosides with the  $(\text{Na}^+ + \text{K}^+)$ -ATPase has been approached from two points of view, namely the potency of enzyme inhibition and the stoichiometry of binding. As mentioned previously the inhibition of enzyme activity shows good correlation with the inotropic effect that is achieved with these drugs. That is to say, noncardiotonic glycosides do not inhibit the enzyme, and furthermore only the cardiotonic glycosides compete for this membrane receptor (Matsui and Schwartz, 1967; Matsui and Schwartz, 1968; Schwartz *et al.*, 1968; Albers *et al.*, 1968). In addition it has been reported from kinetic studies that dissociation of the ouabain-enzyme complex may be related to species sensitivity (Allen and Schwartz, 1969; Tobin and Brody, 1972). At present these observations cannot be used to further our understanding of the mechanism of action of the beneficial or of the toxic effects of these drugs.

Quantitative measurements of binding data are markedly handicapped by the absence of a specific reference component. Consequently, stoichiometric values have been derived with respect to the phosphorylated intermediate (Albers *et al.*, 1968; Jorgensen, 1974a), the enzyme hydrolytic activity (Albers *et al.*, 1968; Jorgensen, 1974a,b), and the sub-macromolecular structure of the  $(\text{Na}^+ + \text{K}^+)$ -ATPase (Kyte, 1972a; Lane *et al.*, 1973; Hokin *et al.*, 1973; Jorgensen, 1974). A further weakness



in such attempts at quantification is the possibility of non-specific binding and the species variation which may occur. Nevertheless some attempts have been made to overcome these difficulties and a brief discussion of the findings is pertinent to this dissertation.

A number of workers have relied on enzyme purification to overcome the aforementioned problems. Despite the use of different methods of purification, results from their laboratories show good correlation. Irrespective of the source of the enzyme, two major polypeptides have been identified as components of the purified enzyme macromolecule (Kyte, 1972a,b; Lane *et al.*, 1973; Hokin *et al.*, 1973; Jorgensen 1974). The larger of these two chains is reported to have a molecular weight of about 95000 as determined by SDS-polyacrylamide-gel electrophoresis (Kyte, 1971a,b; Kyte, 1972b; Hokin *et al.*, 1973; Lane *et al.*, 1973; Jorgensen, 1974; Uesugi *et al.*, 1971), and 139000 by gel filtration (Kyte, 1972b). This polypeptide carries the site for phosphorylation during ATP hydrolysis (Alexander and Rodnight, 1970; Uesugi *et al.*, 1971; Kyte, 1971a,b; Avruch and Fairbanks, 1972; Collins and Albers, 1972; Hart and Titus, 1973b). Ouabain must also bind to this subunit, since it is known to bind to the  $E_2$ -P complex. By examining the reactivity of the sulfhydryl groups Hart and Titus suggested that the binding sites for  $Na^+$  and  $K^+$  may also reside on the large polypeptide (Hart and Titus, 1973).

The smaller subunit is a sialoglycoprotein with a molecular weight of about 55000 (Kyte, 1971; Uesugi *et al.*, 1971; Kyte, 1972a,b; Lane *et al.*, 1973; Hokin *et al.*, 1973; Hokin, 1974). This smaller polypeptide has not been linked to any catalytic function.





Nevertheless it does undergo purification in parallel with the larger polypeptide, the catalytic unit. It is believed that the two polypeptides exist in some mass ratio which has not yet been established. Some of the values quoted for this ratio are 1.7 (Kyte, 1972b), 1.9, (Lane *et al*, 1973), 2.3 (Hokin *et al*, 1973), and 2.8 (Jorgensen, 1974), and the discrepancies may be due to the variation in the preparative procedures and/or the determination of the size of the polypeptide and/or the species from which the enzyme was derived.

The information obtained from ouabain binding studies cannot be collated easily, as it is often varied and contradictory. In 1968, Lindenmayer *et al*, demonstrated the presence of the E-Pi-Ouabain (E-Pi-O) complex, and suggested that its conformation was different from that of the  $E_2$ -P-O complex. Currently this view is also held by other workers (Hart and Titus, 1973a; Hart and Titus, 1973b; Akera *et al*., 1974; Schwartz *et al*., 1974). The suggestion implies that the receptor species is the same, and a number of observations would support this view. For example, the level of drug bound in the presence of different ligands did not result in different values (Inagaki *et al* 1974). Furthermore, dissociation of the complexes with unlabelled drug was also the same (Schon *et al*., 1970, Lane *et al* 1973) and finally, the activation energies derived from rate constants for the binding of ouabain to these two enzyme-phosphate complexes were not different at 21 and 23 kcal/mole respectively (Wallick and Schwartz, 1974).

In contrast to the work of Inagaki *et al*. (1974), several workers have reported that the maximal drug bound to the two complexes is not the same but higher in the  $E_2$ -P-O form (Schwartz *et al*., 1968; Albers *et al*., 1968; Skou *et al*., 1971).





Studies have also been undertaken to examine the relationship between drug binding and specific activity. As the specific activity of the enzyme is increased, the number of binding sites also increase. This correlation between maximal binding and specific activity, although supported by work from a number of different laboratories (Schwartz *et al.*, 1968; Albers *et al.*, 1968; Hansen, 1971; Erdmann and Schoner, 1973b), is not unchallenged. Reports also claim that the capacity of the enzyme for ouabain binding and  $E_2$ -P formation cannot be correlated (Matsui and Schwartz, 1967; Matsui and Schwartz, 1968; Schwartz *et al.*, 1968; Albers *et al.*, 1968).

Attempts have also been made to study the effects of ouabain binding to preparations subjected to lipase treatment. Some reports show a linear correlation between loss of specific activity and ouabain binding, but others reject this correlation. Goldman and Albers (1973) found that after treatment of the enzyme from eel electroplax membranes with phospholipase-A (PPL-A) the specific activity and the ouabain binding capacity decreased in parallel if the  $E_2$ -P-O was the complex estimated. Erdmann and Schoner (1973b) using ox brain  $(Na^+ + K^+)$ -ATPase found that under conditions favourable to  $E$ -Pi-O formation PPL-A caused a loss of the catalytic activity, both the  $(Na^+ + K^+)$ -ATPase and the phosphatase ( $K^+$ -pNPPase, E.C.3.1.1.4), but the ouabain binding and  $^{32}P$  labelling of the membranes remained. If the membranes were treated with phospholipase-C (PPL-C) however, the ouabain binding was not impaired in parallel with the specific activity (Goldman and Albers, 1973).

From the aforementioned information, it would seem likely that the protein component or receptor macromolecule is a single species



as was suggested by Schwartz' group (1968), however the site of ouabain binding probably differs in the  $E_2$ -P-O and the E-Pi-O complexes. These two sites may be maintained by different conformations of the protein. It is not clear at present just how extensive these differences might be. However it is certain that the protein macromolecule is the same for both, and the differences may reside at the tertiary and quaternary levels of organisation. Some information has been gained from the effects of potassium on the binding of ouabain to membrane preparations containing  $(Na^+ + K^+)$ -ATPase. In particular, these studies have attempted to assess the occurrence of conformation changes in the reaction sequence of the enzyme. Robinson (1970) proposed a  $K^+$ -directed conformational change at the step involving the cleavage of the enzyme-phosphate bond. In a subsequent publication Charnock *et al.* (1971) also postulated a  $K^+$ -induced conformational change to facilitate release of Pi from the enzyme macromolecule. Akera and his co-workers (1974) showed quite definitely that  $K^+$  has a distinct influence on the dissociation of the two complexes.  $K^+$  stabilizes the  $E_2$ -P-O complex but not the E-Pi-O complex. In apparent contrast,  $K^+$  also reduces the rate of ouabain binding to  $E_2$ -P. They proposed that  $K^+$  induces a conformational transition which alters the ouabain site thereby decreasing its affinity for ouabain. This  $K^+$ -induced conformational change is therefore independent of dephosphorylation. They also suggest that there may be two different conformational states or a single state with two different ouabain binding sites, which lead to formation of the  $E_2$ -P-O or the E-Pi-O complex.

The following is a summary of the preceding discussion:

- 1) The  $E_2$ -P-O and the E-Pi-O complexes are similar in some respects and very different in others.



- 2) Increasing specific activity of membrane preparations may or may not be correlated with increased formation of  $E_2\text{-P-O}$ .
- 3) The loss of specific activity by treatment with PPL-A may or may not correlate with decreased formation of  $E_2\text{-P-O}$ , but does not correlate with  $E\text{-Pi-O}$ . When the membranes are treated with PPL-C, the loss of specific activity is not accompanied by a parallel loss of  $E_2\text{-P-O}$  formation.

This summary emphasizes the complexity of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  macromolecule. While it is clear that it is capable of undergoing conformational changes (at least two of which have been established), little direct biophysical evidence exists to support this view. Each conformer is endowed with an assortment of binding sites for the different ligands associated with the enzyme function. Furthermore these binding sites may influence each other. This is especially true of the sites for  $\text{Pi}$ ,  $\text{K}^+$  and ouabain. Finally, the phospholipids of the bilayer in which the macromolecule is embedded may participate in one or more of the conformations. This involvement of the lipid is the topic of the following discussion.

#### 4. Lipid involvement

Arrhenius plots (Arrhenius, 1889) of data obtained from enzyme function are often used to demonstrate the thermal sensitivity of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . Such a plot of the reciprocal of the absolute temperature versus the logarithm of the enzyme activity usually results in a discontinuous line with a break at a so-called "critical temperature" ( $T_c$ ) near  $20^\circ\text{C}$ . Above the critical temperature the slope of the line is less than below it. Thus the enzyme has a lower energy of activation above  $T_c$  and higher energy of activation below it.

In order to understand the discontinuities referred to above,





researchers have generally directed their investigations towards a better understanding of the qualitative and quantitative characteristics of the phospholipids associated with this enzyme. A vast amount of information has accumulated and it is discussed under the four following sub-headings:

- a) Reactivation of delipidated membranes with respect to specific activity as well as phospholipid specificity.
- b) Analysis of the phospholipids of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enriched membranes.
- c) The effects of temperature and/or lipid depletion on the partial reactions.
- d) Reconstitution of lipid-free solubilized  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enzyme preparations into liposomes.

a) Lipid depletion is generally achieved by treatment with lipases, detergents or organic solvents. Of the lipases A,C, and D, phospholipase-A (PPL-A) has been used most often. Amongst the detergents, the nonionic detergent Lubrol-WX and the anionic detergent deoxycholic acid (DOC) are most commonly employed. Organic solvent extraction is relatively rarely used.

Ohnishi and Kawamura (1964) are credited with being the first to demonstrate the implication of a lipid in the mechanism of action of this enzyme, and from their work, they concluded that phosphatidyl serine might be the essential lipid.

Since then a variety of phospholipids have been used to restore the hydrolytic activity to the lipid depleted enzyme. Taniguchi and Iida (1972) achieved reconstitution of a PPL-A treated enzyme with phosphatidyl serine (PS) and with phosphatidyl inositol (PI). Hokin





and Hexum (1972) confirmed this work and extended it to include phosphatidic acid (PA) and didodecyl phosphate as compounds capable of reactivating the enzyme activity. These workers also showed that the loss of activity after treatment with PPL-A was not due to lysophosphatides. Using rabbit kidney cortex, <sup>1</sup>Charnock *et al* (1973) compared the effects of temperature on enzyme enriched membranes after treatment with nonidet-P 40 a nonionic detergent, DOC an anionic detergent, phospholipase-C (PPL-C) from *Clostridium Welchii*, and bee venom PPL-A. Both detergents and PPL-C showed a small effect on the slope of the line at the lower temperatures, but the discontinuity in the Arrhenius plot remained, and the critical temperature was similar to that observed with untreated membranes. Treatment with PPL-A however, abolished the discontinuity, and this resulted in a single straight line having a unique slope distinct from the earlier values for  $E_{a_1}$  and  $E_{a_2}$ . Addition of PS to this phospholipase treated enzyme restored the bend, which also occurred at the original  $T_c$ . The work also included an examination of the temperature sensitivity of the  $Mg^{++}$ -ATPase, the temperature sensitivity of this enzyme was neither biphasic nor linear but curvilinear. Furthermore, this curvilinear plot remained unchanged after treatment with detergents or with lipases. We concluded that the  $Mg^{++}$ -ATPase and the  $(Na^+ + K^+)$ -ATPase are distinct entities even though they may be closely associated in the membrane. We also suggested that the lipids associated with the two enzyme were not the same. The detergent studies showed that although the specific activity could be varied considerably,

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<sup>1</sup>Charnock, J.S., Cook, D.A., Almeida, A.F., and Rebecca To. (1973) Arch. Biochem. Biophys. 159, 393. This work will not appear either in the methods section or in the results section. For completion, a copy is included in the appendix.



the resultant enzyme preparations with an increased number of active centres did not demonstrate any significant change in the thermodynamic parameters as determined by temperature studies. The results from studies with PPL-A confirmed those of others (Ohnishi and Kawamura, 1964; Taniguchi and Iida, 1972; Hokin and Hexum, 1972); the data did not permit any unequivocal statement regarding either the specificity of PS or its location in the membrane matrix with respect to the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ .

In addition to PS, PI and PA as lipids capable of restoring the activity and the thermal sensitivity to the lipid depleted enzyme, other compounds have also been used successfully. Kimelberg and Papahadjopoulos (1972) and Palatini *et al* (1972) independently and concurrently reported reconstitution with phosphatidyl glycerol (PG). It is of note that although PC has been used to reactivate delipidated enzyme preparations, it is generally less effective than any of the others that have been used (Kimelberg, 1976). The one exception is the report by Tanaka and Strickland (1965), but this has been criticised on the grounds that the PC was contaminated with PS (Fenster and Copenhaver, 1967). Finally it must be noted that there is a requirement for cholesterol if lipid depletion is effected by the use of organic solvents (Noguchi and Freed, 1971; Jarnefelt, 1972). Thus it would appear that although some lipid is essential for activity a specific phospholipid may not be crucial for enzyme function. In a recent review, Kimelberg (1976) has tabulated the lipids that have been used in reactivation studies. From this table it is apparent that reactivation is most often achieved with PS, and the possibility that this phospholipid participates in some specific manner must still be





entertained. Nevertheless it is certain that removal of lipid from  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  results in a corresponding loss of enzyme activity (Erdmann and Schoner, 1973b; Stahl, 1973), and this loss can be restored to varying degrees by the addition of phospholipid. Complete removal of lipid leads to an irreversible loss of activity (Hegyvary and Post, 1969; Roelofsen *et al*, 1971; Erdmann and Schoner, 1973b).

The most direct evidence for the involvement of lipids in  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  has been obtained by the use of physical methods employing membrane probes. In 1973 Grisham and Barnett employed a lipid spin probe, to examine the effects of temperature on the membrane lipids. This method which reports fluidity changes in the membrane showed a biphasic response to temperature irrespective of whether the probe was located in microsomes or liposomes made from lipid extracts of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . Moreover the values for critical temperature ( $T_c$ , cf. p.17) were also identical in these two situations and similar to the  $T_c$  determined from an Arrhenius plot of enzyme specific activity. Thus it appears that a phase transition of the membrane lipids is directly involved in the discontinuity observed in Arrhenius plots of specific activity. Since the activation energy for hydrolytic function is increased at temperatures below  $T_c$ , it is tempting to suggest that below  $T_c$ , the lipids hinder the function of the protein macromolecule. In a similar approach Charnock and Bashford (1975) employed fluorescent probes and were able to confirm the findings of Grisham and Barnett (1973). Furthermore, they avoided the criticism of probe interference in membrane function, by duplication of the liposome study using a light scattering technique. Thus it is currently well accepted that  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  has an absolute requirement for some phospholipid(s).





From the varied results that have been discussed, it is not possible to sustain or reject the concept of a specific phospholipid as essential for reactivation of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity. Roelofsen and Van Deenen (1973) have listed discrepancies that could accommodate some of the variation, and these have been listed below:

1. The phospholipases used for lipolysis may have been impure, and would consequently lack substrate specificity.
2. The phospholipids used for reactivation may have been impure.
3. Information on residual lipids in the delipidated membranes was absent or sparse; when present the lipids were not characterized.
4. Both the degree of inactivation and reactivation was often neglected.

Although these criticisms certainly apply in a number of instances they do not offer a complete explanation for the discrepancies found in the literature.

Kimelberg and Papahadjopoulos (1974) who also noted discrepancies approached the problem differently. After delipidation of rabbit kidney membranes with DOC they monitored the specific activity and the  $T_c$  on the addition of phospholipids which varied both in head group and chain length. Their results showed that PS and dioleoyl-PG, two phospholipids with unsaturated chains produced the highest level of reactivation, PS being the better of the two. In contrast dimyristoyl-PG and distearoyl-PG succeeded only partially in restoring activity. Their temperature study revealed that the control membrane preparation had a  $T_c$  of about  $30^\circ\text{C}$  which is somewhat higher than that frequently reported by other workers. The delipidated preparation showed no discontinuity in the temperature-activity relationship, and the addition of PS to these membranes introduced a  $T_c$  at  $15^\circ\text{C}$ . Dioleoyl-PG acti-



vated the delipidated preparation which maintained its linear appearance albeit with a lower activation energy. The effect of chain length was clearly demonstrated by reactivation with dimyristoyl-PG, dipalmitoyl-PG and distearoyl-PG: the longer the methylene chain the higher the  $T_c$ . They concluded that a specific requirement for PS and PG was strongly indicated. They also noted that the discontinuities reflected by the enzyme hydrolytic activity did not correspond with the endothermic transitions as determined by differential scanning calorimetry. This is an important observation in the application of physical techniques to biological investigation.

From the data in that paper an interesting observation can be made. Since the delipidated enzyme retained activity, it presumably also retained some lipid. If the retained lipid is the critical lipid then a further addition of other lipids should not have altered  $T_c$ . If residual detergent is not a factor in these findings, then another interpretation must be considered; that is, the closely bound lipids are not uniquely and exclusively responsible for the discontinuity in the temperature activity relationship, but the lipids in the membrane matrix may also be determinants of this phenomenon. This introduces the question as to the role of phospholipids in the function of  $(Na^+ + K^+)$ -ATPase and their contribution to the thermally induced discontinuity.

The phase transitions observed on cooling lipid bilayers has been extensively examined. Although the involvement of the methylene chains of the phospholipids has been known for over a decade (Chapman *et al.*, 1967), a clear understanding of phase transitions in molecular terms is still not possible. In a recent review Lee (1977) reminds us that above the transition the arrangement of methylene chains is still



uncertain, while below the  $T_c$  the methylene chains become stiff and lie parallel to each other and perpendicular to the plane of the membrane. In the case of biological membranes, phase changes due to temperature alteration are also frequently reported but again are poorly understood. The presence of numerous lipid species in a biological membrane introduces the possibility of multiple phase transitions and/or separations. Thus, any discussion dealing with thermal effects necessitates caution. In the case of the  $(Na^+ + K^+)$ -ATPase, the thermally induced phenomenon is definite but whether it is a consequence of a phase separation or transition or both is not known. Three points have been well substantiated:

- i) A change in the membrane occurs on cooling.
- ii) This change usually occurs at about  $20^\circ C$  and is dependent on lipid(s).
- iii) The temperature induced lipid change alters the function of the enzyme protein, resulting in a higher activation energy for ATP hydrolysis.

b) By comparison with the activation experiments, lipid analysis of  $(Na^+ + K^+)$ -ATPase preparations would appear to be a relatively unpopular approach towards the understanding of the involvement of phospholipid in the mechanism of this enzyme. This approach is based on the logic that since purification leads to a concentration of the protein moiety any closely bound lipid would also be concentrated. The most exhaustive study in this area is the work by Wheeler and his co-workers (Wheeler *et al.*, 1975; Wheeler and Walker, 1975; Wheeler, 1975). They studied two methods of enzyme purification, namely DOC-treatment and a sequential NaI-Lubrol extraction. With both preparations they





showed a marked correlation between specific activity and the phospholipid content, when expressed per unit protein. In an examination of the nature of the phospholipids they did not detect a selective loss or concentration of any particular kind of phospholipid in either the DOC-treated membranes or the Lubrol-treated membranes. It is noteworthy that an untreated preparation and a DOC-treated preparation differed slightly in phospholipid composition even though the latter was fifty-three times as active. The more pure preparation showed a small decrease in PC and some increase in lysophosphatides. Wheeler and his coworkers have suggested that although phospholipid specificity by headgroup could not be excluded as a requirement for ATP hydrolysis, additional specificity by fatty acyl chain might exist. In a separate communication (Walker and Wheeler, 1975) circumstantial evidence for this view was provided by an examination of the fatty acyl chains of two phospholipids (PC and PS) which are known to differ markedly in their potential for reactivation (Palatini *et al.*, 1972; Kimelberg, 1976). PS, the more active of the two had 15% C-20 and 83% C-18 chains while PC contained no C-20, 65% C-18 and 35% C-16 chains.

This work supports the suggestion made earlier by Tanaka and Teruya (1973). Their analysis of the phospholipid fatty acyl chains of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  preparations from the brains of the bull frog and the ox showed a preponderance of unsaturated long chains of 18 or 20 carbon atoms. They concluded that the physical state of the enzyme was a decisive factor, and that its conformational changes might be dictated by an activating lipid ligand.

More recently Palatini *et al* (1977) concluded from their work that a requirement for fluid chains and negatively charged polar head





groups existed to "assemble in an expanded lamellar configuration".

Thus it is apparent that the analytical approach towards the elucidation of the lipid involvement is also incomplete. Phospholipids having acidic head groups and long unsaturated fatty acyl chains are most suited to the reactivation of delipidated  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  preparations. At the other extreme however, PS and cholesterol ester have been shown to increase with purification of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  (Kawai *et al.*, 1973) and sulfatide and enzyme content have been shown to increase in parallel in the salt gland of ducks that had been fed a high salt diet (Karlsson *et al.*, 1971).

c) Studies of lipid interactions in the partial reactions have also made use of delipidation procedures. In 1971 Taniguchi and Tonomura treated ox brain microsomes with bee venom PPL-A and studied the enzyme-phosphate intermediate. They suggested that since the formation and the breakdown of this complex were both reduced by treatment with PPL-A, therefore these two stages required some lipid. Since PPL-A does not directly act on the protein moiety of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , it is implied that the contribution of the lipid is towards maintaining a particular conformation(s). Goldman and Albers (1973) using membranes from *Electrophorus electrophorus* found data to support the observation made by Taniguchi and Tonomura (1971). Furthermore they also suggested that the conformational change from  $\text{E}_1\text{-P}$  to  $\text{E}_2\text{-P}$  as well as the final step, the  $\text{E}_2$  to  $\text{E}_1$  conversion were both lipid dependent. This concept of an all embracing effect of the lipid is not shared by Barnett and Palazzotto (1974). Focussing their attention on the effects of temperature, they showed that neither the terminal catalytic step, the so



called  $K^+$ -pNPPase nor the binding of  $[^3H]$ -ouabain to these preparations showed any discontinuity in the Arrhenius plot. Therefore, the authors conclude, these two partial reactions are not lipid dependent. Using both approximations and presumptions, they developed an argument which "leaves the conversion of  $E_2-K$  to  $E_1$  as the most likely site of action of the phase transitions". Because of the weakness of their argument it is not pertinent to compare the suggestion made by Barnett and Palazzotto (1974) with the works cited earlier (Taniguchi and Tonomura, 1971; Goldman and Albers, 1973); there is however one advantage to the approach taken by Barnett and Palazzotto namely, that it permits the study of temperature effects on untreated membranes.

<sup>1</sup>Charnock *et al.*, (1975) used an approach which compared Arrhenius plots of specific activity obtained by ouabain inhibition of the enzyme with those obtained by cation activation. Rabbit kidney membranes subjected to six different treatments were examined: DDC, nonidet P-40, sodium iodide, phospholipase-C, and phospholipase-A alone and after addition of phosphatidyl serine. Our studies showed that above  $T_c$  the  $Ea_1$  did not differ appreciably whether obtained by cation activation or by ouabain inhibition. Below  $T_c$  however there was a measurable difference. For example with the untreated membranes  $Ea_2$  from cation activation data was 60% of the  $Ea_2$  from ouabain inhibition data. This trend was most marked in data obtained from membranes treated with sodium iodide, a chaotropic agent which disrupts lipid-protein and protein-protein interactions in biological membranes

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<sup>1</sup>Charnock, J.S., Almeida, A.F., and Rebecca To. (1975) Arch. Biochem. Biophys. 167. 480-487. This work will not appear either in the methods section or in the results section. For completion, a copy is included in the appendix.





(Hamaguchi and Geiduschek, 1962; Hatefi and Haustein, 1969). The results obtained from PPL-A treated membranes was also noteworthy. With cation activation the discontinuity of the Arrhenius plot was retained. This is in contrast to the ouabain inhibition data as was also found previously (Charnock *et al.*, 1973). In general, all the treatments indicated that cation activation of the enzyme was less influenced than ouabain inhibition. The suggestion was made that the phase transition of the lipids influences the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enzyme protein, and this effect was much more apparent when ouabain inhibition was examined rather than cation activation of the enzyme. We made a further suggestion from the unique effects of PPL-A on the membrane. Since the apparent lysis of PS affected ouabain inhibition and not cation activation, the possibility now existed that this phospholipid generally or in a specific location imparted cardiac glycoside sensitivity to the enzyme. This latter suggestion contrasts with the claim by Barnett and Palazzotto (1974) that ouabain inhibition of the enzyme yielded a linear Arrhenius plot.

Wheeler's group (1975) studied the partial reactions of this enzyme using rabbit kidney membranes that had first been treated with NaI and then partially delipidated with Lubrol-W. They found that Na-dependent phosphorylation was partially inhibited by delipidation but K-dependent dephosphorylation was totally inhibited. They offer the interpretation that the partial reactions up to and including  $E_1 - P$  formation are either not dependent on lipid, or dependent on tightly bound lipid. By contrast phospholipid was essential for the transformation of the  $E_1 - P$  to  $E_2 - P$  and subsequent dephosphorylation. Thus this latter work finds common ground with all of the four works dis-





cussed before it. From the literature then it appears that the  $E_2 - P$  conformer through its formation and/or breakdown is the pivotal point that links the lipid-protein interactions, the conformation changes and the thermal phenomenon of this enzyme. In this regard, Rivas *et al.* (1972) showed that when the tritiated drug-enzyme complex  $E_2 - P - O$  was extracted with chloroform-methanol, the organic phase contained a hydrophobic protein that carried the  $^3H$ -ouabain. Because of the denaturing effects of organic solvents however, this work is usually received with more caution than attention.

d) In recent years some workers (Goldin and Tong, 1974; Hilden *et al.*, 1974; Goldin and Sweadner, 1975; Hilden and Hokin, 1976; Anner *et al.*, 1977) have succeeded in introducing lipid depleted highly purified  $(Na^+ + K^+)$ -ATPase proteins into homogeneous liposomes of known phospholipid composition. The  $(Na^+ + K^+)$ -ATPase-loaded liposomes show coupled catalytic and cation pumping activity and also display sensitivity to ouabain. There is some question as to the efficiency of pumping and to the ion specificity. For example, Goldin and Tong (1974) (Goldin and Sweadner, 1975) observed that the pumping of  $Na^+$  was accompanied by chloride pumping which they suggested was unique characteristic of kidney  $(Na^+ + K^+)$ -ATPases. Anner *et al.* (1977) however could not support this view with their data. They suggested that the method of purification could alter the final outcome and that renal  $(Na^+ + K^+)$ -ATPase as compared with the enzyme from other organs, might be more susceptible to the manipulations administered during the process of purification.

Employing the phospholipid exchange technique of Warren *et al.*, (1974a,b), Hilden and Hokin (1976) were able to obtain a  $(Na^+ + K^+)$ -ATPase



preparation in a pure phosphatidyl choline matrix, showing that their enzyme preparation from dogfish shark rectal salt gland can function in the absence of other phospholipids. They also claimed that  $\text{Na}^+$  and  $\text{K}^+$  pumping was now twice as effective as was observed with endogeneous phospholipids. However this cannot be taken at face value for at least one important reason. The optimal activity of an enzyme preparation necessitates the determination of an optimal temperature of operation. In lieu of the involvement of lipids in the temperature sensitivity of this enzyme, any alteration of the lipid species can only make such comparisons of activity approximate at the best.

Although such reconstitution experiments will have a place in the investigative history of the pump-enzyme, they have contributed very little towards an understanding of its mechanism of action or its interaction with the membrane phospholipids. The following points stand out:

- i) The difficulty of orientation of this macromolecule within the bilayer persists. This problem has special significance for a macromolecule with the defined vectorial properties of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . It is possible that orientation of the macromolecule also has a requirement for lipid.
- ii) The sodium to potassium pumping ratio of 3:2 has yet to be established conclusively. This stoichiometry has special significance for a pump that must maintain a transmembrane electrochemical gradient.
- iii) The optimum temperature of the pumping mechanism for the Hokin reconstituted enzyme was  $25^\circ\text{C}$  (Hilden and Hokin, 1976). This may reflect a suboptimal lipid-protein interaction.



iv) Finally it is a telling point that the reconstitutions which include five different enzyme sources (dog and lamb kidney, beef heart and brain, and *Squalus acanthias* rectal gland) have all been achieved with phosphatidyl choline, and not with phosphatidyl serine, a lipid known to be more effective in reactivation studies (Palatini *et al.*, 1972; Kimelberg, 1976). Presumably this is not for want of trying but from failure to obtain this result.

Perhaps in the biological sense synthetic rather than reconstituted pump-enzyme would be a more appropriate definition. However, semantic improprieties must not stand in the way of the credit that is due to the workers in this field. They have conclusively demonstrated an extremely important point which has concerned the critics of this enzyme from the days of its first description by Skou in 1957. There can no longer be any doubt that the enzyme macromolecule is indeed the 'Sodium Pump' of mammalian cells.

### Rationale

From the introduction it is apparent that neither the clinical nor the biochemical pharmacology of the cardiac glycosides can as yet be adequately explained at the molecular level. However the working hypothesis that the pump-enzyme ( $\text{Na}^+ + \text{K}^+$ )-ATPase is itself the drug-receptor, provides the basis for direct experimental investigation.

This project utilizes an *in vitro* approach to study the ( $\text{Na}^+ + \text{K}^+$ )-ATPase enzyme at the molecular level.

The rationale for this particular work is based on two facts. First, phospholipid is essential for the catalytic function of the en-





zyme, and second, catalytic function is uniquely sensitive to temperature. From this it can be argued that a change in the catalytic function of enzyme enriched membranes, as for example after treatment with detergents or lipases, may be due to qualitative and/or quantitative alteration of the phospholipids. One method of study of this interaction is the Arrhenius analysis of the temperature-activity relationship of the system. For example, after treatment with detergents or lipases, an overall change in membrane fluidity as it affects enzyme activity may be detected. Thus these experiments should permit us to obtain a better understanding of the effects of membrane fluidity on enzyme function and enzyme inhibition by cardiac glycosides.

This will be achieved by first exposing the enzyme enriched membranes to treatments that are known to effect the specific activity of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , followed by an examination of the effects of temperature on the three parameters listed below:

- 1) The catalytic activity (ATP hydrolysis).
- 2) The binding of ouabain to the system.
- 3) The fluidity of the membrane matrix as reported by an ESR probe intercalated in the bilayer.



## METHODS



## 1. General

Distilled water, passed through a battery of deionizing columns was used to prepare all aqueous solutions. The reagents were of Analar grade or better, depending on what was available. The suppliers have been listed in an Appendix III. After the solutions had been made to volume, they were filtered through a millipore filter of pore size  $0.8\mu$ .

## 2. Enzyme preparation

### 2.1 Solutions

Homogenizing buffer contained 250mM sucrose, 30mM L-Histidine (base), 5mM  $H_4$ -EDTA adjusted to pH 6.8 with 1000mM Tris-base.

Wash buffer contained 20mM Tris-base and 1mM  $H_4$ -EDTA brought to pH 7.6 by the addition of 1000mM HCl.

Sucrose buffer contained 275mM sucrose, 0.20mM  $H_4$ -EDTA, and was brought to pH 7.6 with Tris-base.

### 2.2 Enzyme preparation

Ox brain (bos taurus) was obtained fresh from the abattoir and chilled in crushed ice while it was still warm. The cortex was cleared of large blood clots and rapidly frozen in liquid nitrogen, in 200g portions for storage in a freezer at  $-20^{\circ}\text{C}$ .  $(Na^{+} + K^{+})$ -ATPase enzyme preparations were made from frozen or fresh ox brain, according to the general procedure of Charnock and Post (1963).

The frozen tissue was left at room temperature to warm but was not allowed to thaw. While still frozen it was cut into small pieces of about 1g and then allowed to thaw in homogenizing buffer at  $0^{\circ}\text{C}$ .





The thawed tissue, in a ratio of not less than 1g tissue to 10ml buffer was homogenized. Homogenization was carried out at 4°C using either (a) four strokes of a teflon-glass homogenizer, or (b) one 10 s pulse of a Polytron Homogenizer (Brinkmann Instruments, Canada Ltd.) fitted with a PT-20 generator and operated at setting 8. All subsequent steps were performed at 4°C.

Cellular debris was removed by centrifugation at 1000 x g for 15 min in a refrigerated Sorvall RC2-B centrifuge fitted with a SS34 rotor. Further centrifugations were performed at 9000 x g for 20 min to remove possible contamination from mitochondrial particles. A heavy microsomal pellet was isolated from the 9000 x g supernatant by centrifugation at 46000 x g for 30 min, then washed twice by resuspension in wash buffer, and centrifugation again at 46000 x g. The washed pellets were resuspended in sucrose buffer, and stored at -20°C after rapid freezing in liquid nitrogen. The protein content of these microsomal suspensions was determined by the method of Lowry *et al.*, (1951) as described below. Protein concentrations ranged from 2 to 8 mg/ml.

### 2.3 Treatment of the enzyme with deoxycholic acid (DOC)

Treatment with this detergent was carried out by mixing equal volumes of ( $\text{Na}^+ + \text{K}^+$ )-ATPase enzyme suspension (1mg/ml wash buffer) and DOC (2mg/ml wash buffer). After allowing the reaction to proceed for the appropriate time, it was stopped by the addition of an equal volume of ice cold wash buffer. The DOC-treated membranes were recovered by sedimentation at 46000 x g for 30 min. This residue was washed and stored in a manner identical to that described for the untreated membranes. Three different DOC treatments were employed:

- i) 0.1% DOC for 10 min at 4°C.



- ii) 0.1% DOC in the presence of 3mM ATP for 30 min at 30°C.
- iii) 0.05% DOC in the presence of 2mM ATP, 5mM  $\text{MgSO}_4$  and 80mM NaCl for 5 min at 30°C.

Results from preliminary experiments (see Appendix IV) showed that method (ii) was the most appropriate, and it was used routinely thereafter. Unless stated otherwise all DOC-treated membranes were purified by exposure to 0.1% DOC in the presence of 3mM ATP for 30 min at 30°C.

In a separate experiment [Carboxyl- $^{14}\text{C}$ ]-DOC was used to estimate the residual detergent in an enzyme preparation. The sodium salt of DOC was supplied as 150  $\mu\text{l}$  of an aqueous solution containing 2% ethanol (Amersham/Searle). The solution contained  $9.62 \times 10^{-4}$   $\mu\text{moles}$   $^{14}\text{C}$ -DOC with a specific activity of 52  $\mu\text{Ci}/\mu\text{mole}$ . The radiochemical was made up to 104 ml with 0.2% DOC pH 7.6. Thus the working solution contained 0.2004% DOC and 0.003% ethanol and had a specific activity of  $1.1 \times 10^6$  dpm/ml.

In all experiments where ATP was used in the purification process, the membranes were first preincubated at the required temperature along with the ATP for a few minutes to permit binding of the nucleotide to the enzyme, following which the DOC was added.

#### 2.4 Treatment of the enzyme with sodium dodecyl sulfate (SDS)

Treatment with SDS was carried out by mixing equal volumes of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enzyme suspension, 1 mg/ml in wash buffer containing 8  $\mu\text{moles}$  ATP per ml with 0.1% SDS at 30°C. After allowing the reaction to proceed for the appropriate time, it was stopped by the addition of an equal volume of ice cold wash buffer. The SDS-treated membranes



were recovered by sedimentation at 46000 x g for 30 min. This residue was washed and stored in a manner identical to that described for the untreated membranes. From the preliminary experiments (see Appendix V), 30 min exposure to 0.05% SDS in the presence of 4mM ATP at 30°C was chosen as a routine purification procedure thereafter.

In a separate experiment  $\text{SD}^{35}\text{S}$  was used to determine the residual detergent in the enzyme preparation. Lauryl sulfate, sodium salt [ $^{35}\text{S}$ ] was supplied as a dry powder (New England Nuclear). It was dissolved in 4 ml distilled water and 1.0 ml of this stock solution together with 0.8 ml 5% SDS were made up to 50 ml with Tris-EDTA buffer to give a 0.1% solution of  $\text{SD}^{35}\text{S}$  with a specific activity of  $5.55 \times 10^6$  dpm/ml.

## 2.5 Treatment of the enzyme with phospholipase-A (PPL-A) and reactivation with phosphatidyl serine (PS).

The procedure for treatment of the particulate enzyme with PPL-A and subsequent reactivation with PS was adapted from that of Imai and Sato (1960). The  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  containing membranes, 1 mg protein/ml were warmed to 37°C in a medium buffered to pH 7.4 containing sucrose, 275mM, serum albumin 7.35 mg/ml, Tris-base, 14.7mM, 2-mercaptoethanol 3.37mM, EDTA, 0.035mM, and  $\text{CaCl}_2$ , 5.6mM. The reaction was started by the addition of bee venom PPL-A (Sigma Chemical Co.) 1180 units per mg  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  protein, and allowed to continue for 12 min. The reaction was terminated by the addition of 0.67 volumes EDTA 57mM-Tris 321mM, followed by immediate dilution to 3 volumes with ice cold distilled water. The treated membranes were sedimented at 77,500 x g for 3 hr in a Beckman L-3 ultracentrifuge Type 60TI. The pellets





were washed and resuspended as described for untreated membrane.

Reactivation after PPL-A treatment was achieved by incubating equal volumes of PPL-A-treated membranes 1 mg/ml and PS liposomes, at 37° for 10 min. The liposomes were prepared by sonicating 3 mg PS per ml sucrose buffer in a Cole-Parmer Ultrasonic cleaner Model 884S-4 at about 35° till the solution was clear.

## 2.6 Organic solvent extraction and cholesterol determination

Membrane aliquots were diluted 10-fold with distilled water and sonicated for 1 min in a Cole-Parmer Ultrasonic Cleaner, Model 884S-4 and then sedimented at 48000 x g for 10 min in a Sorvall RC 2B centrifuge. The supernatants were assayed for protein content. The pellets were transferred into weighed test tubes with MeOH, and CHCl<sub>3</sub> was added to obtain a 1:2 ratio of the respective organic solvents. The mixture was again sonicated for 1 min and then centrifuged in a bench top centrifuge, model 8845-4. The clear supernatant was poured off and the pellets re-extracted twice more in the same way. The collected supernatants were evaporated in a stream of air and the residual lipid dried under vacuum in a dessicator containing silica. This lipid extract was redissolved in CHCl<sub>3</sub> and stored at -20° until it was used.

The cholesterol content of the lipid extracts was determined by a modified Libermann-Burchard reaction (Huang *et al.*, 1961). Aliquots of the organic solvent extract were evaporated to dryness in glass tubes equipped with teflon lined screw caps. The residue was shaken with 100 µl glacial acetic acid and warmed in a water bath to 25°. The colour reagent composed of 150 ml acetic acid, 300 ml acetic anhydride, 50 ml sulfuric acid (96%), and 10 g anhydrous Na<sub>2</sub>SO<sub>4</sub>, 5 ml per tube. The colour was allowed to develop for 25-30 min and the



absorbance at a wavelength of 632nm was determined in a Beckman DBG spectrophotometer. The optical density was read against a standard curve derived with cholesterol dissolved in acetic acid.

## 2.7 Determination of enzyme specific activity and enzyme protein concentration

All enzyme preparations were routinely assayed for both ouabain sensitive and ouabain insensitive ATPase activity at 37°. In some instances, in particular when the effects of temperature on [<sup>3</sup>H]-ouabain binding were studied, a full temperature profile of ATPase was also constructed.

Enzyme activity was measured by the coupled optical assay system which continuously monitors the oxidation of NADH at 340 nm (Schoner *et al.*, 1967; Schwartz *et al.*, 1969). A Gilford 2400 recording spectrophotometer equipped with a jacketed ethylene glycol/water constant temperature bath was used in these experiments. The temperature of the spectrophotometer cell was controlled at  $\pm 0.2^{\circ}\text{C}$ . Reactions were performed in 100mM glycylglycine (pH 7.6) containing 2mM Mg SO<sub>4</sub>, 80mM NaCl, 20mM KCl and 250mM sucrose which was added to prevent protein settling. The assay ingredients included 3.14mM phosphoenol pyruvate (sodium salt) and 64 units of pyruvate kinase, 19 units lactic dehydrogenase and 0.2 mg NADH in a final volume of 3 ml. The membrane enzyme preparation was added to a temperature equilibrated cuvette and allowed to equilibrate for a further minute before the reaction was initiated by the addition of ATP to a final concentration of 1.5 mM. Monitoring of NADH oxidation commenced immediately after mixing of the cuvette contents with a small plastic swizzel stick. Mg<sup>++</sup>-ATPase activity was followed in the pre-





sence of 0.4mM ouabain, and was subtracted from the total ATPase activity obtained in the presence of Mg, Na, and K to give the ouabain sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase activity.

For the estimation of the ( $\text{Na}^+ + \text{K}^+$ )-ATPase protein by the Lowry assay (Lowry *et al.*, 1951), a standard curve of not less than 5 points was constructed for each protein assay using bovine serum albumin. The absorbance of the samples was read at 650 nm on a Beckman DB-G spectrophotometer or a Gilford Micro Sample spectrophotometer Model 300N.

## 2.8 Arrhenius plots

The Arrhenius equation provides a means whereby the energy of activation of a reaction can be determined. In graphic form ( $\log K$  versus  $1/T$  °K), the Arrhenius plot illustrates the sensitivity of the reaction to temperature. The slope of the line derived from such a plot provides the energy of activation of the reaction.

A number of biological reactions do not conform to the Arrhenius equation and generate a non-linear plot between 37°C and 5°C. The most serious problem in the application of Arrhenius plots to biological systems, is a determination of the number of discontinuities. During the early work in this laboratory (Charnock *et al.*, 1971), considerable importance was attached to the possibility that a visual assessment might introduce bias against a linear slope. Consequently a technique of Bogartz (1968) was employed to fit two lines to a cloud of points. The point of intersection of the two lines was obtained and defined as the "point of inflection" or  $T_c$ . Furthermore an analysis of variance was applied to the two lines above and below the  $T_c$  to ensure that they did not deviate significantly from linearity. These





operations were incorporated in a computer programme that would display the data graphically, as well as calculate the value for  $T_c$  and the values for the two activation energies above and below this inflection (Cook and Charnock, in press). For the correct application of this programme, it is essential that the readings for each replicate be obtained at the same temperature point (i.e. constant values for the abscissa) and that some numerical manipulation can be applied to similarly standardize the values for the ordinate. The latter was achieved by setting the value for the rate obtained at  $37^\circ\text{C}$  as 100% and expressing all subsequent rates obtained at lower temperatures in these terms.

This method was thus most appropriate for deriving the parameters described above in those experimental protocols where a differentiation between linear and non-linear plots was of prime importance. The method has not yet been developed to the level where it can be applied under circumstances where more than one inflection point might exist.

A part of the results presented in this work includes results obtained from ESR experiments. These data could not at first be achieved at fixed temperature points. In order to standardize these data, the individual points were first joined by straight lines. From these lines readings for the ordinate were determined at fixed temperatures for each replicate which were then averaged and statistically evaluated. These averaged data were then subjected to the Arrhenius form of presentation, using the computer programme described above.

Subsequent use of a newer and more precisely temperature controlled ESR spectrometer made it possible to obtain data at  $1^\circ\text{C}$  inter-



vals from 37°C to 1°C. These data could not however be subjected to the numerical standardization described above for the hydrolytic activity (i.e. as a % of the value at 37°C which was set at 100%), because the distribution of standard errors was non-uniform. A suitable method was not found whereby the replicate data could be accumulated and subjected to statistical analysis. All the methods that were attempted were found to bias the results in favour of or against some aspect of the data. A more detailed discussion of this problem appears in the chapter on discussion. In order to avoid these problems, the approach used by Ashcroft *et al.* (1977), namely a simple plot of mean values with the ranges versus some ESR parameter has been adopted.

### 3. Ouabain binding

#### 3.1 Solutions

The wash solution contained 100mM glycylglycine, 2mM  $\text{MgSO}_4$ , 80mM NaCl, 0.2mM EDTA, and was adjusted to pH 7.6 with Tris-base. It also contained a concentration of unlabelled ouabain equal to that in the binding medium.

The binding solution consisted of a wash solution containing 2mM ATP, pH 7.6. The binding solution also contained [ $^3\text{H}$ ]-ouabain from New England Nuclear at the desired concentration (usually  $5 \times 10^{-7}\text{M}$ ) with the specific radioactivity maintained between 300 - 400 dpm/pmole ouabain where possible.

#### 3.2 Ouabain binding

The binding reaction was carried out in a jacketed glass vessel of about 2.5 cm internal diameter, connected to a constant temperature ethylene glycol/water bath. The reaction was initiated at time 0





by the addition of 0.1 ml ( $\text{Na}^+ + \text{K}^+$ )-ATPase (100 to 300  $\mu\text{g}$  protein) to 2.4 ml binding solution pre-equilibrated for 5 min to the desired temperature. A magnetic stirrer was used to maintain constant mixing.

A plastic box was constructed to hold six millipore filter funnels equipped with scinter beds. The filter funnels and millipore filters 0.8 $\mu$  pore size, were pre-washed just prior to initiation of the binding reaction with 2 x 2 ml wash solution (maintained at the temperature of the reaction) delivered by an automatic syringe. The pre-wash eliminated [ $^3\text{H}$ ]-ouabain binding to the filters and also maintained a constant suction pressure on all the filters. Binding times were taken to the time when 1 ml aliquots of the reaction medium were applied to the filters. At 37° aliquots were taken at 5 s intervals. At the lower temperatures these sampling intervals were increased so that at 9° the whole operation took 250 s. However at all temperatures samples were removed at much longer intervals, up to 60 min at 9°, so that both rates and equilibrium levels of [ $^3\text{H}$ ]-ouabain could be obtained.

Rates of [ $^3\text{H}$ ]-ouabain binding were determined from the slopes of a regression analysis of the levels of [ $^3\text{H}$ ]-ouabain bound, using the Olivetti program #681009. Assays were always in duplicate and in some cases in triplicate or more. Disagreement between individual assays was always less than  $\pm 5\%$ , and the mean values obtained were very reproducible upon repeated assay of samples stored at -20° for several days. No correction was made for any residual non-specific binding of [ $^3\text{H}$ ]-ouabain to protein, as in the absence of activating ligands, this was found to be negligible in these experiments.

### 3.3 Radiolabelled ouabain and counting procedures

Tritiated ouabain from New England Nuclear was supplied as a





solution in ethanol with a specific activity of 12 Ci/mM. The ethanol was blown off in a gentle stream of nitrogen and distilled water added to give a stock [ $^3\text{H}$ ]-ouabain solution of about 10 pmoles ouabain per ml, and a specific activity of about  $1 \times 10^9$  dpm per ml.

After filtration of the enzyme protein the millipore filters were dried in air, disintegrated in 1 ml MeOH and counted in a fluor of the following composition: Naphthalene 100 g, PPO 5 g, Dioxane 720 ml, and Toluene 142 ml. The samples were counted to 3% error in a Beckman LS-100 liquid scintillator with a 33-38% efficiency.

#### 4. Electron spin resonance

##### 4.1 General comments

Electron spin resonance (ESR) has been used for a number of years to study biological processes. Until 1965 the application of this technique was confined to biochemical reactions involving two phenomena:

- a) the formation of free radicals as for example during enzyme reactions, carcinogenesis or radiation damage, and
- b) the presence of paramagnetic metals as for example in metalloenzymes.

In 1965 Ohnishi and McConnell and Stone *et al.*, introduced the stable free radical and as a consequence the application of ESR to biological investigations expanded rapidly. The extent of this sudden interest is apparent from the many reviews and books that have appeared.

The free radical is usually a nitroxyl group stabilized by incorporation into a ring structure to produce a spin label. The spin



label can be linked to a variety of biological molecules that may be substrate, drug or other molecules, to form spin probes. The spin probes are relatively stable in the normal biological temperature range and in the pH range 3 to 10.

An unpaired electron possesses a spin which gives rise to a magnetic dipole. According to the quantum theory the spin angular momentum of electrons can only occur in integral multiples of a quantum and therefore the magnetic moment associated with the electron must also be quantized. Consequently an unpaired electron when placed in an external magnetic field can assume only two possible orientations; their magnetic dipoles will be aligned either parallel or antiparallel to the external field. The splitting of the allowed energy states of the electron in an applied magnetic field is called the electronic Zeeman effect. More electrons will be in the lower energy state (parallel to the external magnetic field) and the technique of ESR is to induce spin transitions from the lower level to the higher level by the application of electromagnetic radiation (microwaves). The electrons in the upper energy state lose energy through relaxation processes and fall to the lower level. In this way net absorption of energy is maintained.

An unpaired electron in a free radical or spin label molecule will interact with other magnetic dipoles present in the molecule. Since some nuclei within the molecule may possess nuclear spin and hence magnetic dipoles, these nuclei can interact with the unpaired electron and with the applied magnetic field. The orientation of a nuclear spin  $I$  with the magnetic field is quantized into  $2I + 1$  allowed orientations. In each of these nuclear spin states, the interaction



between the electronic and nuclear magnetic moments is different. The interaction of an unpaired electron with a nucleus with spin  $I$  therefore gives rise to a hyperfine splitting of the ESR spectrum into  $2I + 1$  transitions, each of which can be labelled by the nuclear spin quantum number  $M_I$ . In a nitroxyl spin probe, the unpaired electron is localized in the region of the N-O fragment and experiences a hyperfine interaction with the  $^{14}\text{N}$  nucleus (spin  $I = 1$ ) so that the isotropic ESR spectrum consists of three lines whose separations are characterized by the hyperfine splitting constant  $A_0$  (or  $a_0$ ). The field at the centre of the ESR spectrum is a measure of the  $g$ -value (spectroscopic splitting) of the electron in its molecular environment. For nitroxides,  $g = 2.006$ .

A typical isotropic ESR spectrum for a nitroxide is shown in Fig. 1, where the first derivative of the absorption is plotted against the applied magnetic field. The figure also indicates some spectral parameters and their corresponding symbols. Thus,  $h_{+1}$ ,  $h_0$  and  $h_{-1}$  denote the peak heights of the low-, the mid- and the high-field lines while the widths of these lines are denoted by  $W_{+1}$ ,  $W_0$  and  $W_{-1}$  respectively. The subscripts  $+1$ ,  $0$  and  $-1$  refer to the allowed values of  $M_I$ , the nitrogen spin quantum number.

The spectrum shown in Fig. 1 is characteristic of rapidly rotating spin labels. Using some approximations, theories of line widths have been developed for short correlation times (Kivelson, 1960; Freed and Fraenkel, 1963). From these theories an expression has been derived (Raison *et al.*, 1971) which relates the rotational correlation time  $\tau_0$  to measurable peak heights and widths.

$$\text{Thus:} \quad \tau_0 = K W_0 \left[ \left( \frac{h_0}{h_{-1}} \right)^{\frac{1}{2}} - 1 \right] \text{ s}$$





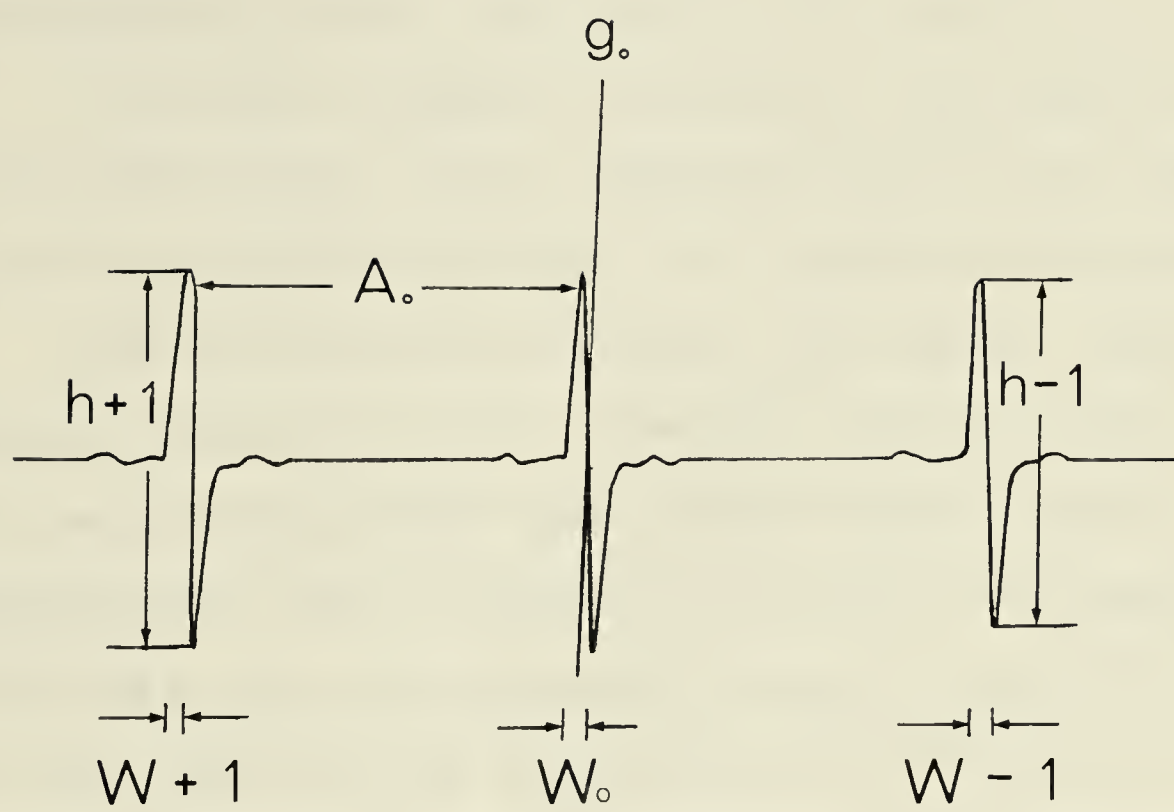


Fig. 1.



where:  $K$  is a constant =  $6.5 \times 10^{-10}$  s for nitroxides as determined from the spectral data of Griffith *et al.* (1965).

$W_0$  is the line width of the centre line and  $h_0$  and  $h_{-1}$  are the peak heights of the mid- and low-field lines (see Fig. 1).

This formula although it incorporates some assumptions and a number of approximations, gives a reasonable measure of the correlation time for freely tumbling spin labels in an isotropic environment.

When nitroxides are restricted in their motion, spectral anisotropy is observed, and the spectroscopic splitting factor  $g$  as well as the splitting constant  $A$  are now both angular dependent. By incorporating spin labels in crystals, the values of the hyperfine splitting and  $g$ -value can be determined (Libertini and Griffith, 1970; Seelig, 1970) when the field is parallel to or perpendicular to the N-O axis of the molecule. These parameters have been illustrated in sub-section 4.7.

## 4.2 Instrumentation

Some of the ESR spectra were obtained using a Varian V-4502 BST spectrometer. This instrument is equipped with an Alpha Model 3039 digital NMR gaussmeter for magnetic field calibration and a Varian-4557 temperature control. The temperature of the sample chamber was examined by a copper-constantin thermocouple to  $0.1^\circ$ . Microwave frequencies were monitored with a HPX 532B frequency meter. In addition, some spectra were obtained using a Bruker B-ER 420 spectrometer coupled to a Varian Magnet VFR-240B. This spectrometer is equipped with a Bruker temperature control unit Model #-ST 100/700, which employs a chrome-alumel thermocouple. This latter unit maintained the temperature of the sample to  $0.1^\circ$  and also permitted the very detailed temperature studies.



### 4.3 Sample tubes

Although quartz glass cells have traditionally been used for esr spectrometry, the application of this physical technique to biological investigation has introduced the use of an assortment of glass capillary tubes including pasteur pipettes, micropipettes, and hematocrit tubes. The obvious advantage of this improvisation is the low cost of these sample tubes which obviates the need for cleansing and reuse. A disadvantage however, is the presence of paramagnetic compounds or metals in the glass. This paramagnetic contamination was observed in the four varieties of glass tube examined, and these are listed below.

- 1) Microhematocrit capillary tubes (Blue-Tips, Plain. Fisher Scientific).
- 2) Microhematocrit capillary tubes (Capilets - Dade. Canlab.).
- 3) Micropipettes (Microcaps. Drummond.).
- 4) Pasteur pipettes (Dispopipets. Fisher Scientific).

The variation in the paramagnetism of these four tubes was not very marked; the microhematocrit tubes from Fisher Scientific, were chosen as they were readily available. By restricting the gain as well as the modulation amplitude, it was possible to restrict the intensity of the contaminant peak to an acceptable level.

The sample tubes were first sealed at one end after which the label was introduced as a solution in methanol. The solvent was blown off in a stream of nitrogen and the label remained as a film on the surface of the glass tube. Liposome or microsome suspensions were then placed in this tube and incubated at 37° for 10 min to permit

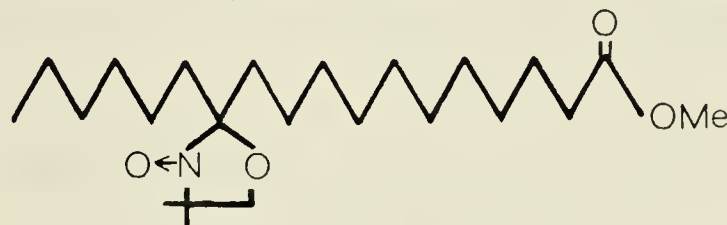




diffusion of the spin label from the glass.

#### 4.4 The spin label

The spin label, N-oxyl-4'-4'-dimethyl oxazolidine-12 keto methyl stearate (M 12-NSE) was a gift from Dr. J.K. Raison, Macquarie University, Sydney, Australia, and its formula is shown below:



The main advantage of this label was that at the time this project was commenced, spin probes of membrane lipids were not commercially available. In addition it had also been tried on biological membranes and found to report from within the lipid bilayer (Raison *et al.*, 1971).

A serious problem in the application of ESR spectroscopy to the examination of biomembranes is the possibility of line broadening due to interactions between the electrons of the label molecules when pooling or clustering of the label occurs (Scandella *et al.*, 1972). In order to avoid this spin-spin coupling effect it was essential to establish a concentration of label that could be employed within the limits of the gain and modulation amplitude that were referred to above. Fig. 2 gives the results of experiments designed to investigate these limits. The contribution from the glass is shown in Fig. 2a and the gain settings on the spectrometer were restricted such that this amount would not be exceeded. The spectrum of 2 nmoles M 12-NSE as a film on the walls of the sample tube is shown in Fig. 2b. This spectrum which was obtained at a similar



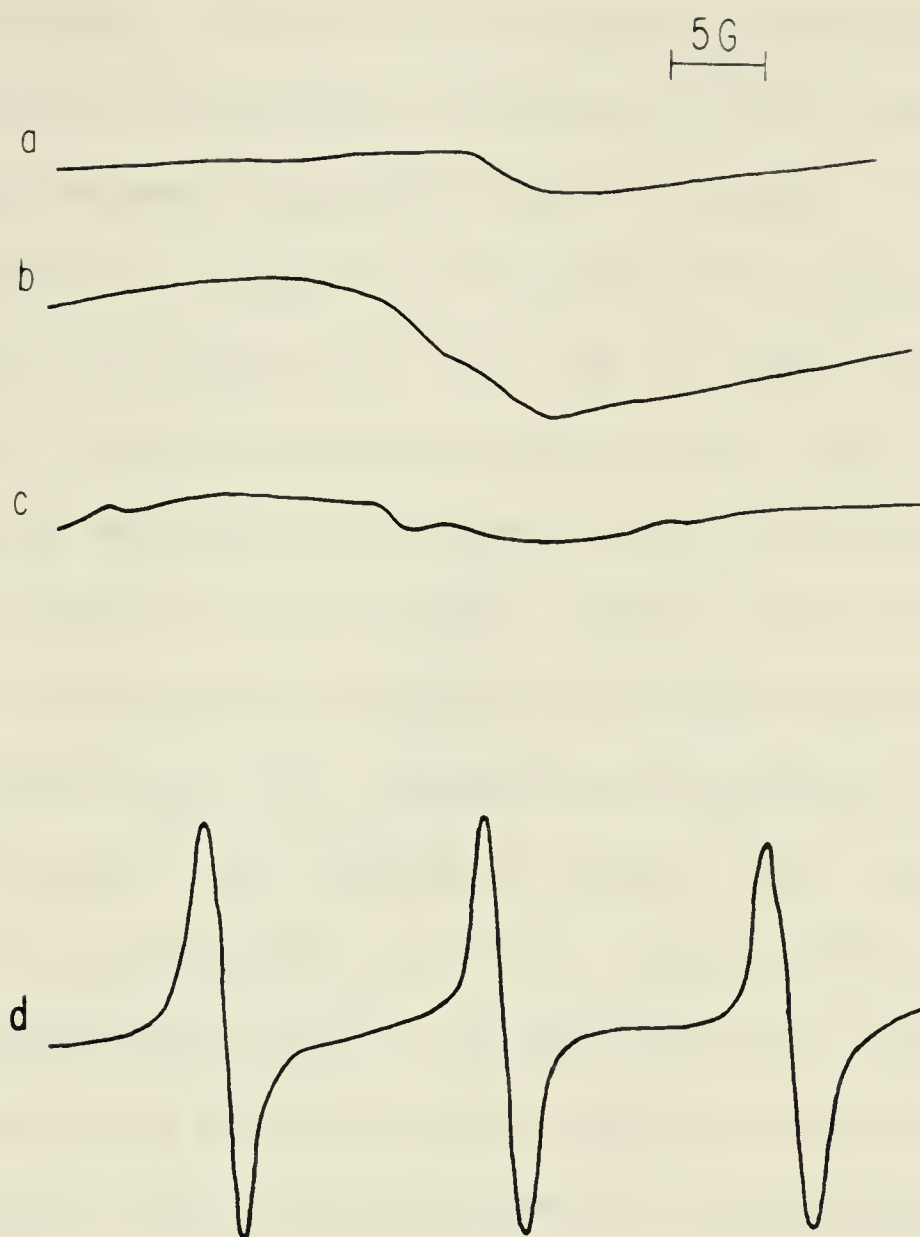


Fig. 2 Limits of detection. The ESR spectra shown were obtained from (a) the empty sample tube; (b) 2 nmoles of M 12-NSE as a film on the surface of the empty sample tube; (c) after the addition of buffered sucrose solution to (b) above; (d) from 0.5 nmoles M 12-NSE in methanol.



gain setting to Fig. 2a shows a broad signal superimposed on the signal recorded from the glass. Together they appear as a distortion of the base line. However, this spectral contribution will be reduced when some of the label becomes incorporated into the membrane. Thus, the base line distortion due to label on the walls of the sample vessel must be less than that shown in Fig. 2b. Fig. 2c shows an examination of the effects of a buffered sucrose solution on this label film. Immediately after the addition of the aqueous phase of the system no change could be detected in the spectrum. However, after 30 min contact, peaks of low intensity were apparent which were superimposed on the broad spectrum of the film. Subsequent more prolonged exposure of up to 24 h did not alter the intensity or shape of these peaks. At this concentration of probe there is little solubilization of the M 12-NSE in the buffered sucrose medium. By comparison when a solution of M 12-NSE in methanol was examined at the same gain settings as those used above, a typical spectrum was obtained but the intensity of the peaks was so great that the concentration of the label had to be reduced four-fold ( $0.5 \text{ nmoles}/25 \mu\text{l}$ ) to accommodate in the Fig. 2b.

From these experiments four important points can be made:

- 1) The contribution from label in aqueous solution is negligible.
- 2) Excess label present as a film on the surface of the sample tube does not make a significant contribution to the spectrum.
- 3) A concentration of 2 nmoles M 12-NSE represents a four-fold excess of label that can be detected at the maximum gain settings to be used.
- 4) The paramagnetic signal in the glass sample tube has been contained.





#### 4.5 DMPC liposomes as control bilayers

It is well known that liposomes made from synthetic phospholipids will incorporate nitroxide spin labels (Butler *et al.*, 1974). In liposomes labelled with fatty acid spin labels, the long axis of the spin probe is oriented perpendicular to the plane of the bilayer (Seelig 1970; Hubbell and McConnell, 1971). Thus the line shape of the spectrum will vary depending on the depth to which the nitroxide reporter group of the label is embedded in the bilayer, and also on the characteristics of the spin probe. M 12-NSE is an ester of 12-nitroxyl stearic acid and is essentially insoluble in aqueous solution. Liposomes made from dimyristoyl phosphatidyl choline (DMPC) have been used as control lipid bilayer system in which to examine the line shape of this spin probe. Liposomes made from a 1.5% suspension of DMPC in sucrose buffer were serially diluted to give 4 different liposome concentrations. Thus when the liposomes were added to sample tubes each containing 2 nmoles M 12-NSE increased ratios of probe:liposome were obtained. Spectra from these various labelled preparations are shown in Fig. 3. It is quite clear that the water insoluble probe does not remain on the surface of the sample tube but diffuses readily into the liposomes. At the lowest probe:liposome ratio there is no significant contribution to the spectrum from label on the glass surface, although this becomes apparent at very high probe:liposome ratios.

All the spectra have broader lines than those obtained from a methanol solution of the probe, clearly showing that when M 12-NSE is embedded in a homogeneous lipid bilayer, its molecular mobility is restricted. This restraint is believed to be due to the packing of the



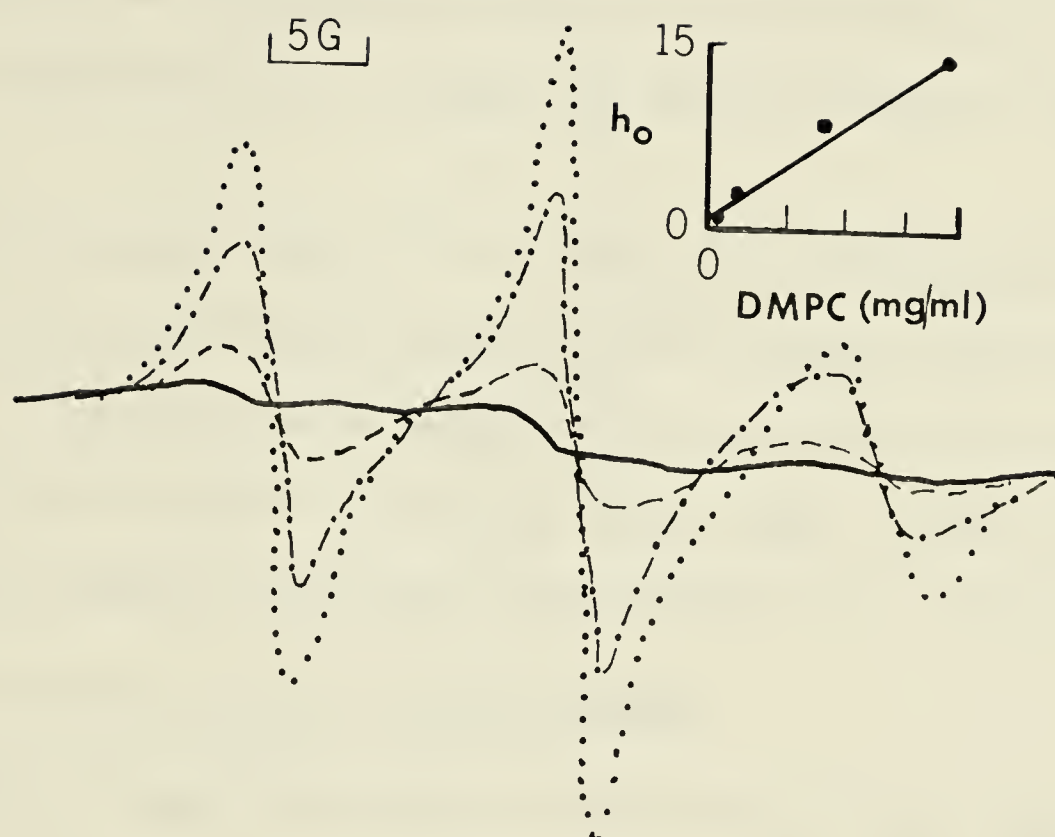


Fig. 3. M 12-NSE in liposomes. The spectra from serial dilutions of DMPC liposomes labelled with M 12-NSE (.....), 5 mg DMPC/ml; (-·-·-), 2.5 mg/ml; (-----), 0.5 mg/ml; (——), .05 mg/ml. Insert: peak height ( $h_0$ ) vs. concentration of DMPC in mg/ml.



fatty acid chains of the phospholipid molecules (Smith, 1972; Keith *et al.*, 1973) of the lipid bilayer. As the liposome concentration is decreased there is a corresponding decrease in the intensity of the signal which is seen as a reduction in signal peak heights, but the line shapes remain relatively similar. Since the probe concentration is fixed at 2 nmoles, the different spectra represent increasing probe:liposome ratios. Thus, over the concentration range examined here, the ratio of probe to liposome does not influence the motional freedom of the nitroxide.

Also shown in Fig. 3, is a plot of the peak heights of the midfield lines ( $h_0$ ) of these spectra and the liposome concentration in mg DMPC/ml. The linear relationship that is apparent again demonstrates that any M 12-NSE present on the glass at the lower liposome concentrations has not made any significant contribution to the spectrum.

#### 4.6 Temperature study of DMPC liposomes

Fig. 4 shows the effects of temperature on spin labelled DMPC liposomes. At 37° the spectrum is essentially isotropic with the three characteristic peaks. The tumbling time calculated with the formula described by Raison *et al.* (1971), which was shown on page reveals  $\tau_0$  to be less than  $5 \times 10^{-9}$ s. If however the liposomes are cooled the motional freedom of the nitroxyl reporter group is markedly affected. The intensity of all three peaks is reduced; in particular at the lowest temperature shown, 16°C the high field peak cannot be measured with sufficient accuracy. Thus the tumbling time as a measure of the change in bilayer fluidity in this system is not appropriate, and alternate measures were sought. This investigation is dealt with in the following section.





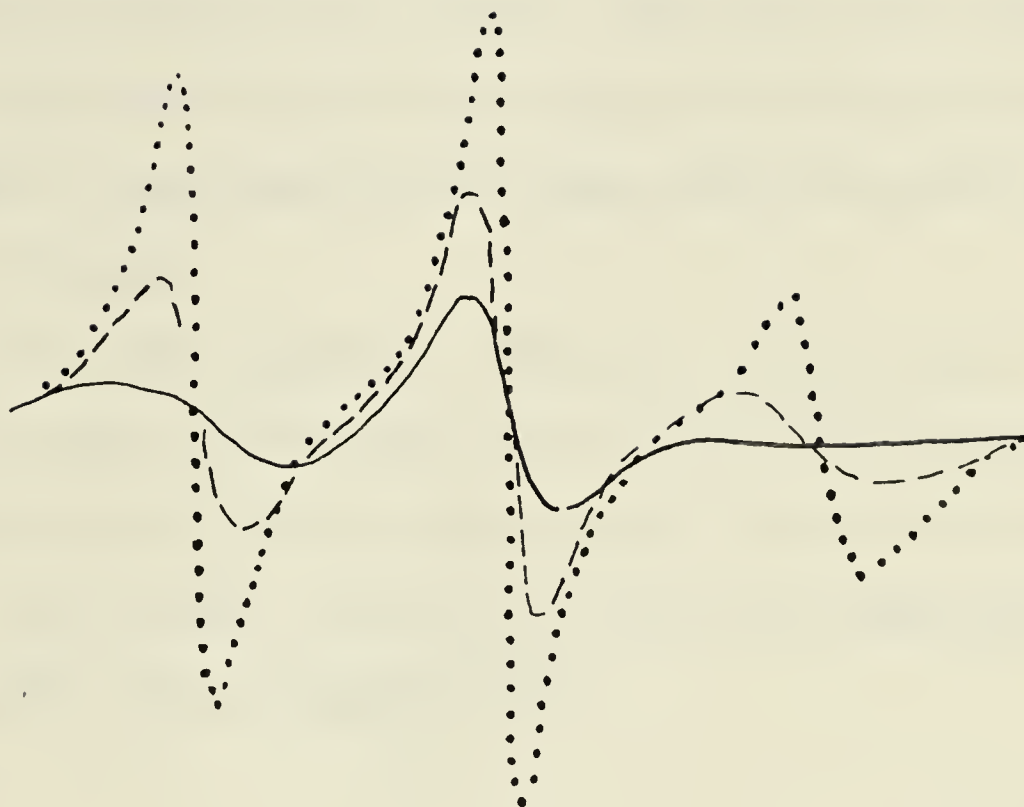


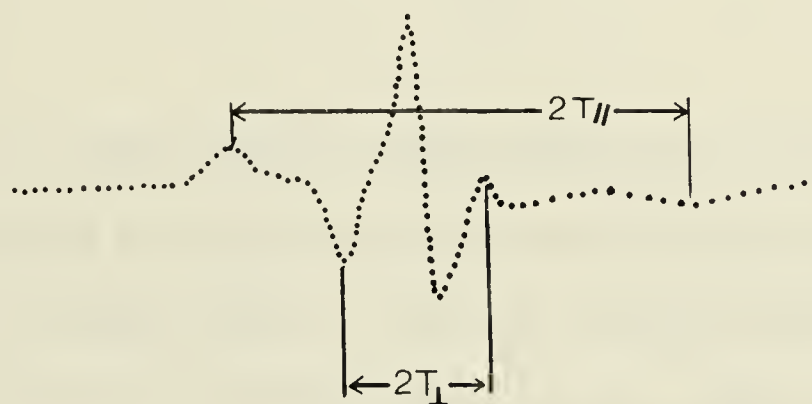
Fig. 4 Effects of temperature on the spectrum of M 12-NSE labelled liposomes. 2.5 mg/ml of DMPC liposomes were labelled as described in text. Spectra were recorded at 37° (• • •), at 20° (— — —) and at 16° (———).



#### 4.7 Quantitative measures

It was mentioned in sub-section 4.1 that isotropic spectra of unrestricted spin labels are frequently quantified by making use of the formulation of Raison *et al.* (1971). This method does not apply to restricted spectra and in such cases a variety of methods have been derived. Some of these techniques are shown below along with the appropriate references.

a) The order parameter  $S$  (McConnell and McFarland, 1970; Seelig, 1970; Hubbell and McConnell, 1971) is most often employed for the quantification of fluidity changes in spin labelled biological membranes. The following illustration is taken from Seelig and Hasselbach, 1971, Eur. J. Biochem. 21:17.



$$\text{Order parameter, } S = \frac{0.56 (T_{//} - T_{\perp})}{a}$$

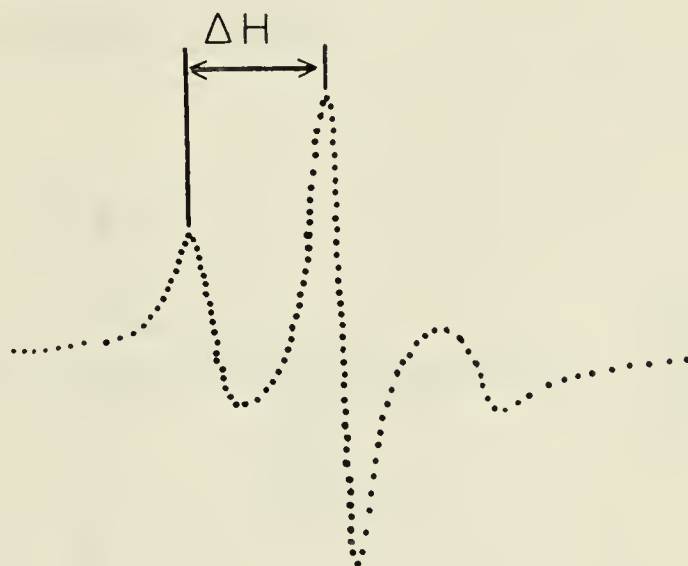
$$a = \left( \frac{2T_{//} + T_{\perp}}{3} \right)$$

Similarly Rotten *et al.*, 1973, Biochim. Biophys. Acta 323: 509, have employed  $2T_{\text{max}}$  and  $2T_{\text{m}}$  which if applied to the illustration above, are alternative symbols for  $2T_{//}$ .

Grisham and Barnett, 1972, Biochim. Biophys. Acta 266:613,

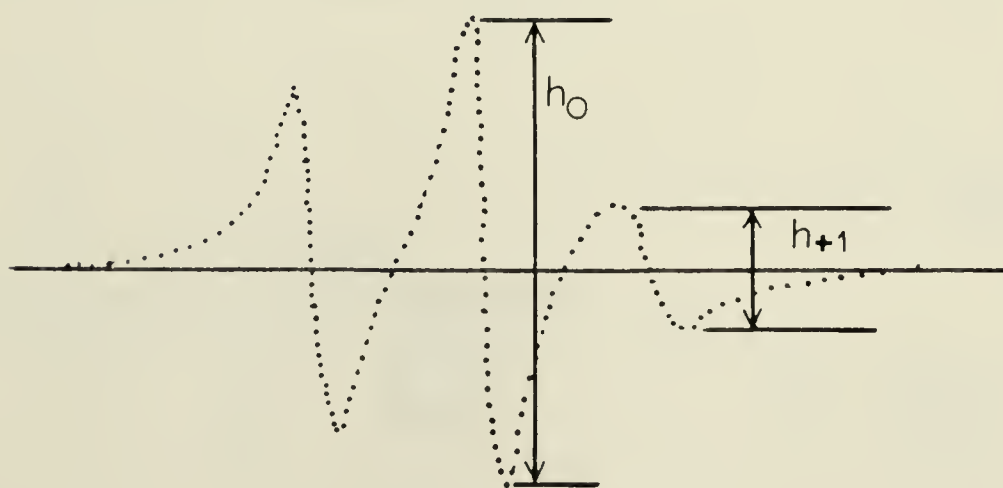


used a parameter they defined as  $H_{\max}$  which is also an alternative symbol for  $2T_{//}$  as shown in the figure above. In a subsequent publication, 1973, Biochim. Biophys. Acta 311:417, these same authors employed another method of quantification suggesting that neither the order parameter  $S$  nor the  $H_{\max}$  could be applied to the experiments being reported. This parameter which was called  $\Delta H$  is shown below:



b) The ratios of peak heights have also found use in the quantification of spectra, and some variations are presented below.

A simple ratio of the mid- and the high-field peak heights, as shown below was used by Hegner *et al.*, 1973, Biochim. Biophys. Acta. 307:452, and also by Eletr *et al.*, 1973, J. Mol. Biol. 78:351.

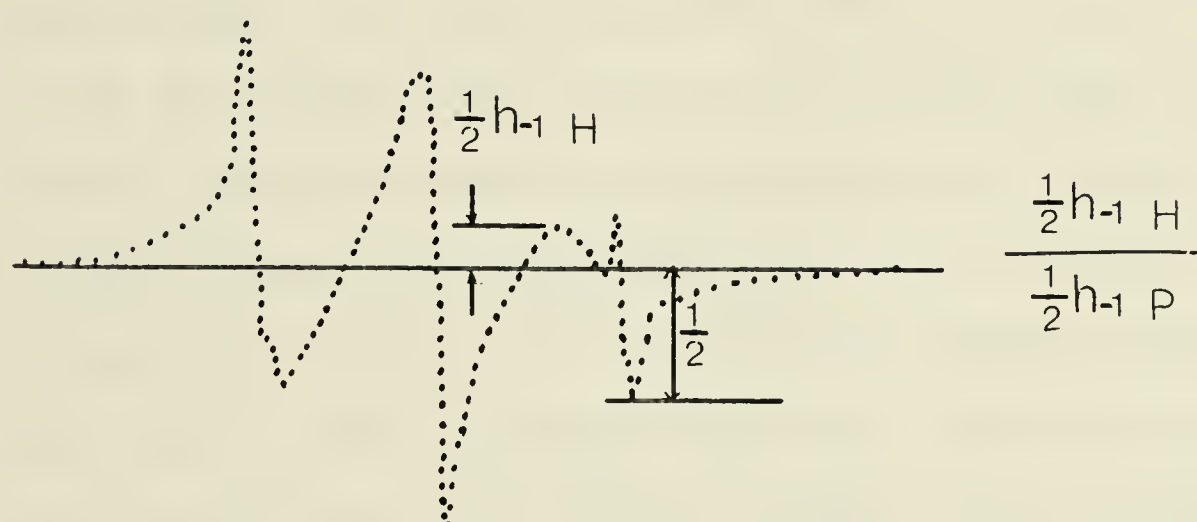


$$\frac{h_0}{h_{+1}}$$

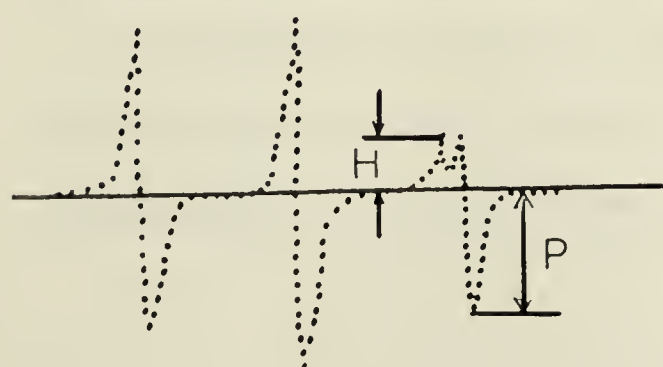




When labelled biological membranes yielded a spectrum with two high field lines, a ratio of the intensity from labels in the hydrophobic region (H) and in the polar region (P) was used. In this approach, half the peak height of the high field line due to labels in the hydrophobic region is denoted by  $\frac{1}{2} h_{-1} H$  and the corresponding value for labels in the polar region is denoted by  $\frac{1}{2} h_{-1} P$ . This method is illustrated below, and is reproduced from Linden et al., 1973, J. Supramol. Struct. 1:523.



A similar method was employed by Grant and McConnell, 1973, Proc. Nat. Acad. Sci., U.S.A. 70:1238 and by Shimshick and McConnell, 1973, Biochemistry 12:2351. The authors define a "Tempo spectral parameter" which is denoted by 'f' and compares the intensity of peaks from spin labels in the hydrophobic region and in the polar region. This method is illustrated below:



f = "Tempo spectral parameter"

$$f = \frac{H}{H + P}$$



From this list it is apparent that a number of factors can contribute to the type of spectrum, which in turn will determine which method of quantification will be appropriate. Six methods of quantification were compared using the effects of temperature on the control bilayer, the DMPC liposome. The results are shown in Table 1.

There is a marked effect of temperature on all three spectral peaks with the high field peak ( $h_{-1}$ ) showing the greatest effect. To compare these different quantitative approaches an inflection point has been derived. This inflection point, ( $T_c$ ), is the point of intersection of the two straight lines that best fit the non-linear data points obtained, using the computer programme described in section 2.9. The purpose of reporting a calculated value for  $T_c$  in Table 1, seeks only to emphasize changes in the slope of plots showing the effects of temperature upon a number of spectral parameters. From the comparison given it can be seen that the different methods of quantification that were used show good correlation. A small difference in the value of  $T_c$  obtained with data from the  $h_{-1}$  peak is noted. Because of the difficulty of obtaining accurate measurement of this peak at low temperatures, the high-field peak is deemed an unsuitable parameter for spectral quantification of temperature effects when M 12-NSE is employed. Thus in the studies to be reported, evaluation of the spectra has been confined to methods that make use only of the mid-field and low-field peaks,  $h_0$  and  $h_{+1}$  respectively. Where the spectrum is typical of a very mobile probe, comparable to a probe solution in methanol, the high-field line has been incorporated into the spectral evaluation.



TABLE 1

The effect of temperature on different spectral parameters of spin labelled DMPC liposomes.

Parameter <sup>a</sup>	40.3° <sup>b</sup>	33.0°	23.1°	19.2°	17.1°	8.6°	T <sub>c</sub> <sup>c</sup>
h <sub>+1</sub>	100	71	42	24	12	7	20.74
h <sub>o</sub>	100	83	60	40	26	17	20.91
h <sub>-1</sub>	100	68	39	18	5	--	19.98
h <sub>o</sub> /h <sub>+1</sub>	100	117	144	169	225	225	20.47
h <sub>o</sub> /h <sub>-1</sub>	100	120	156	226	503	---	18.18
t <sub>o</sub>	100	112	184	309	707	---	20.27

<sup>a</sup> Peak height was measured in mm.

<sup>b</sup> The data obtained at the different temperature points were expressed as a percentage of the value obtained at 40.3° which was set at 100.

<sup>c</sup> As determined by computer programme using 10 temperature values from 17° to 40°.





## RESULTS



## 1. Catalytic activity

The interrelationship between temperature and the hydrolytic function of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  has been examined by a variety of techniques and the following general conclusions were drawn and listed in the introduction (p. 20):

- a) A change in the membrane occurs on cooling.
- b) This change usually occurs at about  $20^\circ\text{C}$  and is strongly influenced by lipid(s).
- c) The temperature induced lipid change alters the function of the enzyme protein, resulting in a higher activation energy for the hydrolysis of substrate ATP.

These general properties have been examined in greater detail for a number of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  preparations. In the first part of this subsection they will be discussed with reference to membranes enriched in  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  prepared from rabbit renal cortex. Essentially this section will make use of the information contained in the two publications that have been reproduced in Appendix I and II.

### 1.1 Rabbit kidney $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$

The early work in this laboratory had been carried out with  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enriched membranes prepared from rabbit renal cortex (Charnock *et al.*, 1971a; Charnock *et al.*, 1971b). This enzyme preparation exhibits the characteristic thermal sensitivity which is frequently demonstrated as a non-linear Arrhenius plot as shown in Fig. 5. The point of intersection occurs at about  $20^\circ\text{C}$  and separates two slopes such that the smaller slope or lower activation energy occurs above this point. Extraction of these membranes with the nonionic detergent



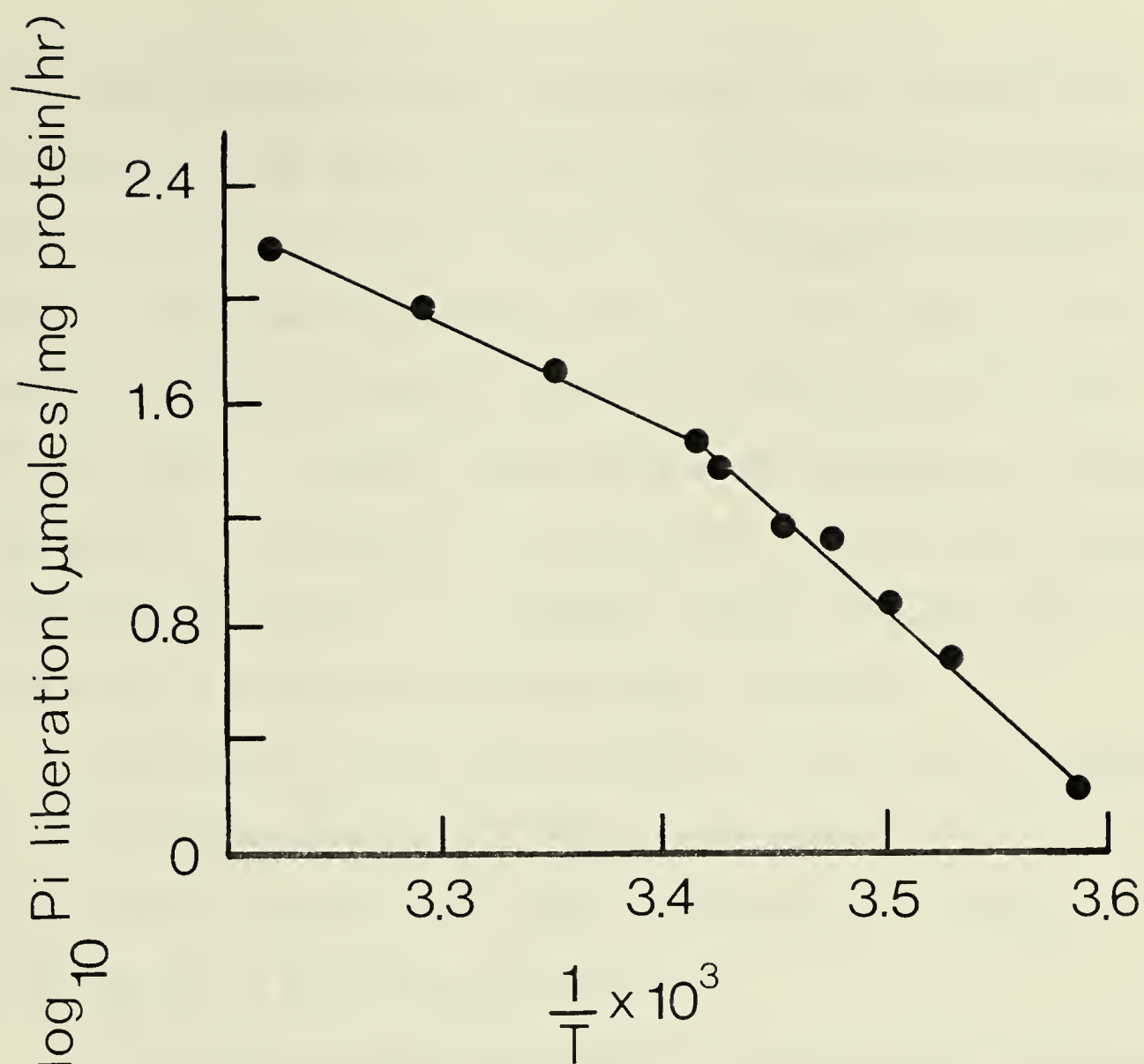


Fig. 5. A typical Arrhenius plot of inorganic phosphate liberation by ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase. The values were obtained as the difference in activity  $\pm 1$  mM ouabain with 80 mM  $\text{Na}^+$ , 20 mM  $\text{K}^+$ , and 4 mM MgATP. Assays were in duplicate. Activation energies above and below the transition temperature are 14.0 and 30.6 kcal/mole, respectively; from Charnock, Cook and Casey, 1971, Arch. Biochem. Biophys. 147, 323-329.





Nonidet P 40 or with the anionic detergent DOC (0.1%) resulted in a marked increase in the specific activity of the preparation. However the inflection point  $T_c$ , was not altered or abolished but remained at about 20°C. Furthermore the values obtained for the apparent activation energies above and below  $T_c$ , the  $E_{a1}$  and the  $E_{a2}$  also did not change appreciably. Therefore the treatment of the enzyme with these two detergents did not result in any detectable change in the temperature dependence of the  $(Na^+ + K^+)$ -ATPase, although in both cases the specific activity of the enzyme was markedly increased.

A variety of lipases are available for the lysis of membrane lipids. Of these phospholipase-A (PPL-A) removes one of the fatty acyl chains and was employed as a means of altering the membrane lipids of the  $(Na^+ + K^+)$ -ATPase preparations.

If the membranes were subjected to lipolysis with bee venom PPL-A the thermal response of the enzyme was markedly altered. The effect of temperature on the specific activity of these membranes when presented in the Arrhenius form could be represented by a single straight line with a slope that was distinct from either  $E_{a1}$  or from  $E_{a2}$ . If however these PPL-A treated membranes were reactivated by incubation with phosphatidyl serine (PS), then the characteristic non-linearity was restored and the values for  $T_c$  as well as the two activation energies that were now obtained were similar to the corresponding control values.

From these studies it was also determined that the  $Mg^{++}$ -ATPase which is frequently associated with the  $(Na^+ + K^+)$ -ATPase in these membrane preparations was quite different in its sensitivity to temperature, and presumably its interaction with the membrane lipids. In no



case was a linear or non-linear Arrhenius plot obtained, the most appropriate description being curvilinear for such a plot. Thus for the purpose of this dissertation, discussion of the  $\text{Mg}^{++}$ -ATPase component of the system has been excluded.

With this information the general conclusions stated above, can now be rewritten as they pertain specifically to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enriched membranes prepared in this laboratory from rabbit renal cortex:

- a) The temperature-activity relationship of the hydrolytic activity of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  containing membranes yields a non-linear Arrhenius plot. Thus the hydrolysis of ATP by this enzyme preparation occurs at two distinct energy levels, represented by two different values for  $E_{a1}$  and  $E_{a2}$ . Above a critical temperature  $T_c$ , of about  $20^\circ\text{C}$  the apparent activation energy ( $E_{a1}$ ) is considerably lower than that ( $E_{a2}$ ) below  $T_c$ .
- b) The non-linearity can be abolished by pretreatment of the membranes with PPL-A, and subsequent addition of PS restores the non-linearity. Thus it follows that the non-linearity is at least partially a lipid dependent phenomenon, and PS plays some role therein. However, it is not implied that PS is unique in this regard, nor is it specifically located in the membrane with respect to the protein macromolecule.
- c) Further enrichment of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  by treatment with the nonionic detergent, Nonidet P 40, or with the cationic detergent DOC does not alter either the critical temperature  $T_c$  or the two activation energies.

The thermal sensitivity of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  was examined further by comparing Arrhenius plots constructed from data obtained by



two different methods, namely activation of the enzyme with cations and the subsequent inhibition of this active state with ouabain. A schematic representation of the methodology is shown below:

- A. Enzyme + Mg + ATP = Enzyme + Mg + ATP + ouabain → Basal hydrolysis.
- B. Enzyme + Mg + ATP + Na + K → Maximal hydrolysis.
- C. Enzyme + Mg + ATP + Na + K + ouabain → Ouabain insensitive hydrolysis.

Therefore, in Method I, Cation Activation = B - A while in Method II, Ouabain Inhibition = B - C.

These two methods of obtaining rates for the hydrolysis of ATP were compared for rabbit kidney membranes treated in six different ways. The results which are summarized in table 2a,b have been discussed in detail in the publications given in Appendix I and II to this dissertation.

For untreated membranes, both methods, cation activation and ouabain inhibition yield non-linear Arrhenius plots with similar inflection points,  $T_c$ . The activation energies obtained by these two methods were not however identical; the values obtained for  $E_{a_2}$  showed a markedly greater difference than the corresponding values for  $E_{a_1}$ . Thus, for the control membranes a comparison of the two methods of determining enzyme activity yields the following observations:

- a) Both Arrhenius plots are non-linear.
- b) The  $T_c$  are similar in both cases.
- c) The  $E_{a_1}$  is slightly higher for the system when assayed by inhibition with ouabain.





TABLE 2a

APPARENT ACTIVATION ENERGY OF  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  AFTER VARIOUS TREATMENTS AS DETERMINED BY OUABAIN

## INHIBITION OF THE ENZYME

Conditions	No. of Experiments	$E_{a1}$ above $T_c^{\circ a}$	$E_{a2}$ below $T_c^{\circ a}$	$T_c^{\circ}$
Untreated control	6	$19.1 \pm 1.3$	$43.1 \pm 3.9$	$16.5 \pm 1.5$
Deoxycholate	6	$17.8 \pm 1.7$	$36.3 \pm 4.2$	$18.5 \pm 2.3$
Nonidet P40	13	$16.4 \pm 0.5$	$37.2 \pm 5.1$	$19.5 \pm 1.8$
Phospholipase A	7	$23.9 \pm 1.5^b$	$24.0 \pm 3.9^b$	-
Phospholipase A and Phosphatidylserine	5	$20.2 \pm 1.1$	$43.6 \pm 7.2$	$18.7 \pm 0.5$
Phosphatidylserine	3	$19.5 \pm 1.2$	$39.7 \pm 1.7$	$20.4 \pm 1.2$

<sup>a</sup>  $E_a$  given as kcal/mole  $\pm$  standard error.<sup>b</sup> Values for  $E_{a1}$  and  $E_{a2}$  are significantly different ( $P < 0.05$ ) from untreated controls.



TABLE 2b

APPARENT ACTIVATION ENERGY OF (Na<sup>+</sup> + K<sup>+</sup>)-ATPase AFTER VARIOUS TREATMENTS AS DETERMINED BY CATION

ACTIVATION OF THE ENZYME

Conditions	No. of Experiments	Ea <sub>1</sub> above T <sub>C</sub> <sup>a</sup>	Ea <sub>2</sub> below T <sub>C</sub> <sup>b</sup>	T <sub>C</sub> <sup>o</sup>
Untreated control	4	15.4 ± 0.7	27.5 ± 1.9	16.7 ± 1.6
Deoxycholate	4	17.5 ± 2.1	30.4 ± 5.9	18.7 ± 1.4
Nonidet P40	4	15.6 ± 0.6	25.6 ± 1.0	19.5 ± 1.2
Phospholipase-A	4	22.0 ± 0.5	36.0 ± 6.7	18.3 ± 2.7
Phospholipase-A and phosphatidylserine	4	21.6 ± 0.5	28.7 ± 1.2	18.7 ± 0.5
Phosphatidylserine	3	17.0 ± 0.7	30.7 ± 1.7	16.2 ± 1.3

<sup>a</sup>Temperature-activity relationship determined by cation activation with 80mM Na<sup>+</sup> and 20 mM K<sup>+</sup>.

<sup>b</sup>Values for Ea<sub>1</sub> and Ea<sub>2</sub> given as kcal/mole ± standard error of the mean.



- d) The  $Ea_2$  is markedly higher for the system when assayed by inhibition with ouabain.

This pattern was also observed with membranes treated with the nonionic detergent Nonidet P 40 and with the cationic detergent DOC. Once again it is emphasized that the values obtained for  $Ea_2$  reflected the greatest difference between cation activation and ouabain inhibition of  $(Na^+ + K^+)$ -ATPase. This finding suggested that below  $T_c$ , ouabain inhibition of the enzyme is more sensitive to temperature changes than activation of the system by  $Na^+ + K^+$ .

The most profound difference in the data obtained by cation activation and by ouabain inhibition is observed with membranes treated with PPL-A. As was observed previously, Arrhenius plots of  $(Na^+ + K^+)$ -ATPase activity determined by ouabain inhibition can now be described by a linear plot with a unique slope. In the case of the cation activation data however, the non-linearity persisted. Moreover, with this latter method, there is also a change in the values obtained for the activation energies but the  $T_c$  remains unchanged. If the PPL-A treated membranes are reactivated by incubation with PS, then the data obtained by the two methods of enzyme assay conform to the general pattern described above, namely, a discontinuous Arrhenius plot results. Under these conditions, the values for  $T_c$  are similar for the two assay methods, and the  $Ea_2$  for inhibition of the enzyme by ouabain is again markedly greater than  $Ea_2$  for cation activation of  $(Na^+ + K^+)$ -ATPase.

In summary, the characteristics listed previously for rabbit renal  $(Na^+ + K^+)$ -ATPase (p. 64) can now be extended as follows:

- a) The temperature-activity relationship of membrane bound





( $\text{Na}^+ + \text{K}^+$ )-ATPase from rabbit kidney cortex which is demonstrated by a non-linear Arrhenius plot over the range 5°C to 37°C, can be determined either by cation activation of the enzyme or by inhibition with ouabain.

- b) The temperature dependence of the enzyme when determined by ouabain inhibition is greater than when determined by cation activation, particularly below  $T_c$ .
- c) Lipolysis of the membranes by PPL-A reveals that the thermal sensitivity of the enzyme is significantly different when determined by ouabain inhibition or by cation activation. With cation activation the characteristic non-linearity of the enzyme is retained but not with ouabain inhibition. This latter effect of PPL-A can be overcome by incubation of the partially delipidated preparation with PS.

Thus it might be suggested that specific interaction between the protein macromolecule and some phospholipid species is associated with, or is directly responsible for the binding of ouabain (cardiac glycosides), but not the binding of sodium or potassium ions to the membrane-enzyme complex. From this work it cannot be claimed that PS alone is unique in imparting this property.

The conclusions drawn from this work emphasized the urgency of an examination of the effects of temperature on the binding of ouabain to this enzyme.

## 1.2 Alternate enzyme source

Preliminary experiments on the binding of [ $^3\text{H}$ ]-ouabain to the ( $\text{Na}^+ + \text{K}^+$ )-ATPase revealed a need for large amounts of enzyme enriched



membranes, and the small yield from rabbit kidney cortex made it imperative that an alternate source of enzyme be obtained. The ox (*bos taurus*) brain was selected as a possible starting material in an attempt to conserve both the preparation time as well as the cost involved. The following section describes the effects of temperature on the  $(\text{Na}^+ + \text{K}^+)$ -ATPase enriched membranes prepared from ox brain, before and after treatment with detergents and with phospholipase-A.

### 1.3 Ox brain $(\text{Na}^+ + \text{K}^+)$ -ATPase enriched membranes<sup>1</sup>

Although the different methods employed for the preparation of ox brain  $(\text{Na}^+ + \text{K}^+)$ -ATPase have been described in detail in the chapter on methods, they are briefly listed below for ease of reading:

Untreated membranes,

four strokes of a teflon-glass homogenizer - method [a]

one 10 s pulse of a Polytron Homogenizer fitted with a PT-20 generator and operated at setting 8 - method [b].

DOC treated membranes,

0.1% DOC for 10 min at 4°C with membranes from [a] and from [b]

0.1% DOC in the presence of 3 mM ATP for 40 min at 30°C with membranes from [b].

0.05% DOC in the presence of 2 mM ATP, 5 mM  $\text{MgSO}_4$  and 80 mM NaCl for 5 min at 30°C with membranes from [b].

SDS treated membranes,

0.05% SDS in the presence of 4 mM ATP for 30 min at 30°C with membranes from [b].

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<sup>1</sup> Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) *Biochim. Biophys. Acta.* 465, 77-92. See Appendix VI.





The  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  preparations obtained by different treatments were first examined for the effects of temperature on catalytic activity. The hydrolytic activity was determined at 8 or more temperature points between  $37^\circ\text{C}$  and  $5^\circ\text{C}$ . Since the specific activity of individual preparations varied within any one treatment group, the following standardization measure was employed: the hydrolysis rates obtained at the different temperatures were expressed as percentages of the rate at  $37^\circ\text{C}$  and averages determined thereof. These data were used to construct so-called mean Arrhenius plots. From these plots, slopes of the lines were derived to give the  $E_{a1}$  and  $E_{a2}$  values, and the  $T_c$  which was obtained from the point of intersection of these lines. As was described in the methods, a computer programme was used to assist in obtaining these mean values. The results are shown in Table 3.

The results obtained with untreated membranes, whether prepared by method [a] or by method [b] are characteristic of this  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enzyme. The Arrhenius plot in both preparations is again non-linear with an inflection point,  $T_c$  at about  $20^\circ\text{C}$ . However the two preparations are not identical with respect to their energies of activation. The less disruptive process, method [a], yields an  $E_{a1}$  of 18.1 kcal/mole and an  $E_{a2}$  of 37.0 kcal/mole, while the corresponding values for membranes prepared by the more disruptive polytron method are 14.6 and 29.8 kcal/mole. The data do not permit interpretation of these differences at the present time. The differences might signify an alteration in the residual membrane phospholipids, consequent to the methods employed for tissue disintegration. The relationship of the two activation energies is also shown in Table 3, and is represented by a ratio of  $E_{a1}:E_{a2}$ . In both cases this ratio is 0.49.





TABLE 3

APPARENT ACTIVATION ENERGY OF OX BRAIN ( $\text{Na}^+ + \text{K}^+$ )-ATPase AFTER VARIOUS TREATMENTS AS DETERMINED BY OUABAIN INHIBITION OF THE ENZYME\*\*

#	Treatment	n	$E_{a1} \pm \text{SE.}$ kcal/mole	$E_{a2} \pm \text{SE.}$ kcal/mole	$\frac{E_{a1}}{E_{a2}}$	$T_c \pm \text{SE.}$ °C	$P <$ ( $E_{a1}, E_{a2}$ )
1	*Untreated	9	$18.1 \pm 1.10$	$37.0 \pm 3.40$	0.49	$20.0 \pm 1.00$	.001
2	Untreated	4	$14.6 \pm 1.95$	$29.8 \pm 0.30$	0.49	$20.9 \pm 1.31$	.001
3	*DOC, 4°C	6	$21.8 \pm 0.15$	$49.2 \pm 3.90$	0.44	$17.8 \pm 0.80$	.001
4	DOC, 4°C	4	$19.7 \pm 1.89$	$41.8 \pm 3.08$	0.47	$17.9 \pm 0.93$	.001
5	DOC + ATP, 30°C	5	$16.4 \pm 0.80$	$45.4 \pm 5.40$	0.36	$16.6 \pm 1.83$	.001
6	DOC + ATP + Na + Mg, 30°C	3	$15.1 \pm 0.93$	$32.8 \pm 5.30$	0.46	$19.2 \pm 1.53$	.05
7	SDS + ATP, 30°C	3	$13.6 \pm 1.68$	$30.2 \pm 1.20$	0.45	$20.0 \pm 1.27$	.01
	MEAN		17.0	38.0	0.45	18.9	
8	DOC, 4°C + PPL-A	3	$26.9 \pm 1.30$	$20.3 \pm 4.20$	1.32	$30.8 \pm 5.00$	.20
9	DOC, 4°C + PPL-A + PS	2	$17.6 \pm 2.10$	$35.9 \pm 3.45$	0.49	$22.7 \pm 2.40$	.05

\* Membranes prepared by method (a), all others by method (b).

\*\* Data taken from Charnock et al., Appendix VI.



Data from the five experiments with detergent treated membranes, (four with DOC and one with SDS) all generate Arrhenius plots that are qualitatively similar and are discussed as a single group. The characteristic non-linearity is again common to this group, demonstrating the presence of two distinct activation energies above and below the  $T_c$ . In all cases the two slopes reflect significantly different energies of activation above and below  $T_c$  ( $P < 0.05$ ). The mean  $E_{a1}$  and  $E_{a2}$  values for the detergent treated membranes of 17.3 and 39.9 kcal/mole compare favourably with the corresponding values of 16.4 and 33.4 kcal/mole for untreated membranes. The values obtained for  $T_c$  fall in a range from 16.6°C to 20.9°C but whether this reflects experimental variability or some specific protein-lipid or other interaction cannot be determined from these data. With one exception, that of PPL-A treatment, the two activation energies of this group display a constant relationship to each other as is apparent from the ratio  $E_{a1}:E_{a2}$ . Furthermore, the mean value for  $E_{a1}:E_{a2}$  of this group, (excluding treatment with DOC + ATP at 30°C) is 0.46, showing close similarity to corresponding values obtained with untreated membranes. Treatment with DOC and ATP at 30°C yields a ratio of  $E_{a1}:E_{a2} = 0.36$ , but as was noted above, such differences may be attributed to experimental variability or membrane alteration with equal confidence.

The effects of phospholipase-A on the  $(Na^+ + K^+)$ -ATPase enriched ox brain membranes are also given in Table 3. The results show that the lipase treated membranes differ from untreated or detergent treated membranes in many respects. The  $T_c$  at 30.8°C is considerably higher than is usually reported for untreated membranes enriched with this enzyme, and the values for the activation energies, 26.9 and 20.3



kcal/mole for  $Ea_1$  and  $Ea_2$  respectively are also distinct. The difference in the slopes of these two lines is also reduced ( $P = 0.20$ ) and the ratio of  $Ea_1:Ea_2$  of 1.32 suggests a possible linear rather than non-linear plot for these data. The inverse arrangement of the slopes implies that as a consequence of phospholipase-A treatment the  $(Na^+ + K^+)$ -ATPase enzyme is now less efficient at the higher temperature. The data presented in this table do not offer an explanation for this unusual observation. The extent of the difference in the behaviour of the delipidated  $(Na^+ + K^+)$ -ATPase implies a profound alteration of the membrane on exposure to phospholipase-A. If the lipase treated membranes are reactivated by incubation with PS, the variation in the values obtained for  $Ea_1$  and  $Ea_2$ , and also for  $T_c$  as compared with untreated membranes, is within experimental error. Thus reversal of these effects with this phospholipid suggests that lipase treatment leads to alteration of the lipids rather than a direct effect on the protein moiety of this enzyme.

From the results described in this section a number of characteristics of ox brain  $(Na^+ + K^+)$ -ATPase can be listed.

- a) The enzyme displays a thermal sensitivity that is typical for the  $(Na^+ + K^+)$ -ATPase.
- b) Under the experimental conditions employed, namely 8 to 10 temperature points from 37°C to about 5°C, an Arrhenius plot of hydrolytic activity presents a discontinuous profile which is best described as having two distinct slopes. The slopes intersect at a so-called 'critical temperature'  $T_c$  of about 20°C. Above this temperature the enzyme has an activation energy of about 17 kcal/mole and below  $T_c$  the requirement is approximately double this value.





- c) If the enzyme is purified by extraction with the cationic detergents DOC or SDS, the temperature-activity relationship is qualitatively unaltered.
- d) Treatment with PPL-A leaves a unique membrane in which the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  protein can still function but with a very different temperature-activity relationship from that found in untreated membrane preparations.
- e) PS reverses the effects of lipase treatment, thereby implicating the membrane phospholipids in the thermal effects.

From this study it is apparent that the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  prepared from ox brain membranes is similar to the enzyme obtained from rabbit kidney cortex. It is concluded that in the context of this project, the ox brain is an adequate substitute for the rabbit kidney cortex as a source of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ .

## 2. Ouabain binding\*

### 2.1 General

As was alluded to in the introduction and the rationale of this thesis, the binding of  $[\text{}^3\text{H}]$ -ouabain to the E-Pi complex was only of marginal interest to this project. Consequently, conditions leading to optimal enzyme phosphorylation, i.e.  $\text{E}_2\text{-P}$  formation were used to study the binding of ouabain to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  from ox brain. Thus

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\*The project to study the binding of ouabain to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  was done in collaboration, and some of the data presented here have appeared in press: "Variation in sensitivity of the cardiac glycoside receptor characteristics of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  to lipolysis and temperature", Charnock, Simonson and Almeida (1977) *Biochim. Biophys. Acta.* 465, 77-92, (see appendix VI). The ouabain binding experiments were carried out by Mr. L.P. Simonson.



all ouabain binding experiments were conducted in the presence of 2 mM  $\text{Na}_2\text{ATP}$ , 2 mM  $\text{Mg}^{++}$  and 80 mM  $\text{Na}^+$ . Fig. 6 shows the results obtained when drug binding is measured in the presence of ATP, the activating cations Na and Mg, and  $5 \times 10^{-7}\text{M}$   $[\text{}^3\text{H}]\text{-ouabain}$ . Under these conditions, the rate of binding is very rapid, and remains linear for only a short time before equilibrium levels are attained. When ATP was omitted the equilibrium level of  $[\text{}^3\text{H}]\text{-ouabain}$  bound to the membranes fell to less than 10%, while in the absence of ATP as well as the cations, there was a further reduction. In the latter situation, the ouabain bound was not significantly different from the background level which was obtained without the addition of enzyme protein. These findings are similar to those of Taniguchi and Iida (1971, 1972).

## 2.2 Effects of drug concentration and protein concentration on the binding of ouabain.

Using the conditions which are optimal for  $\text{E}_2\text{-P}$  formation, namely 2 mM  $\text{Na}_2\text{ATP}$ , 2 mM  $\text{Mg}^{++}$  and 80 mM  $\text{Na}^+$ , binding data were obtained to study the effects of drug concentration on the initial rates of ouabain binding. The results are shown in Fig. 7. From the graph it is apparent that within the range of drug concentrations that were used,  $10^{-8}$  to  $10^{-6}\text{M}$ , the maximum rate of ouabain binding to the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  receptor was not attained. This is in contrast to the findings of Erdmann and Schoner (1974) who demonstrated that in their experiments with ox brain  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  preparations the maximum amount of ouabain binding occurred at a drug concentration of  $5 \times 10^{-7}\text{M}$ . At  $1 \times 10^{-6}\text{M}$ , the highest concentration that we employed the initial rate of binding was too fast to be measured experimentally, in spite of the fact that the



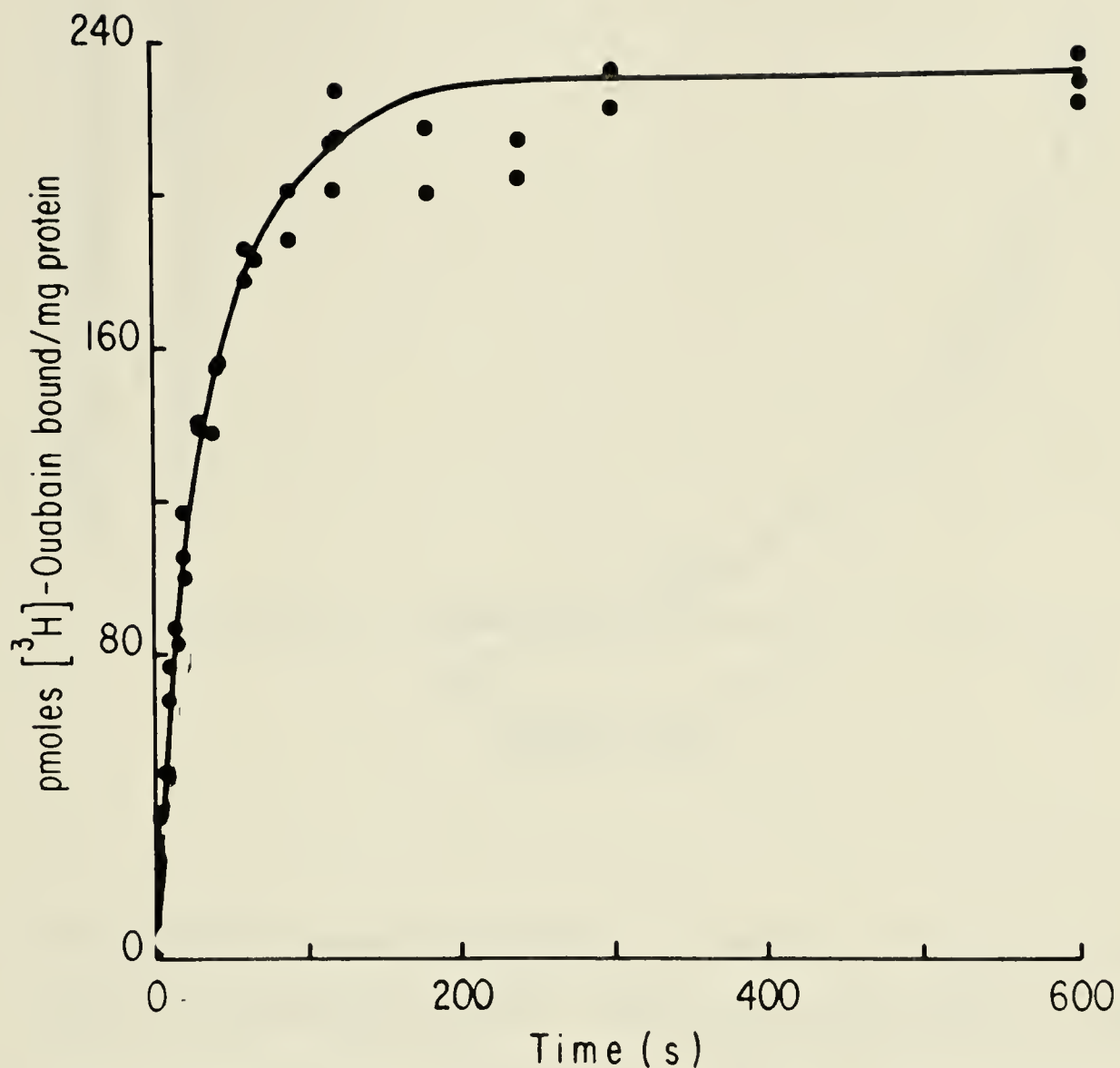


Fig. 6 Progress curve of  $[^3\text{H}]$ -ouabain binding to a membrane preparation of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . The enzyme had been treated with 0.1% DOC at  $4^\circ\text{C}$  for 10 min. The protein content of this preparation was  $128\ \mu\text{g}/\text{ml}$ ; 78% of the total ATPase activity of the preparation was inhibited by  $0.4\ \text{mM}$  ouabain and the ouabain sensitive specific activity was  $38\ \mu\text{moles Pi}/\text{mg protein/hr}$  at  $37^\circ\text{C}$ . The specific activity of  $[^3\text{H}]$ -ouabain was  $424\ \text{dpm}/\text{pmole}$  after dilution with unlabelled carrier ouabain to give a final concentration of  $5 \times 10^{-7}\text{M}$ , with  $2\text{mM Na}_2\text{ATP}$ ,  $2\text{mM Mg SO}_4$  and  $80\text{mM NaCl}$ .





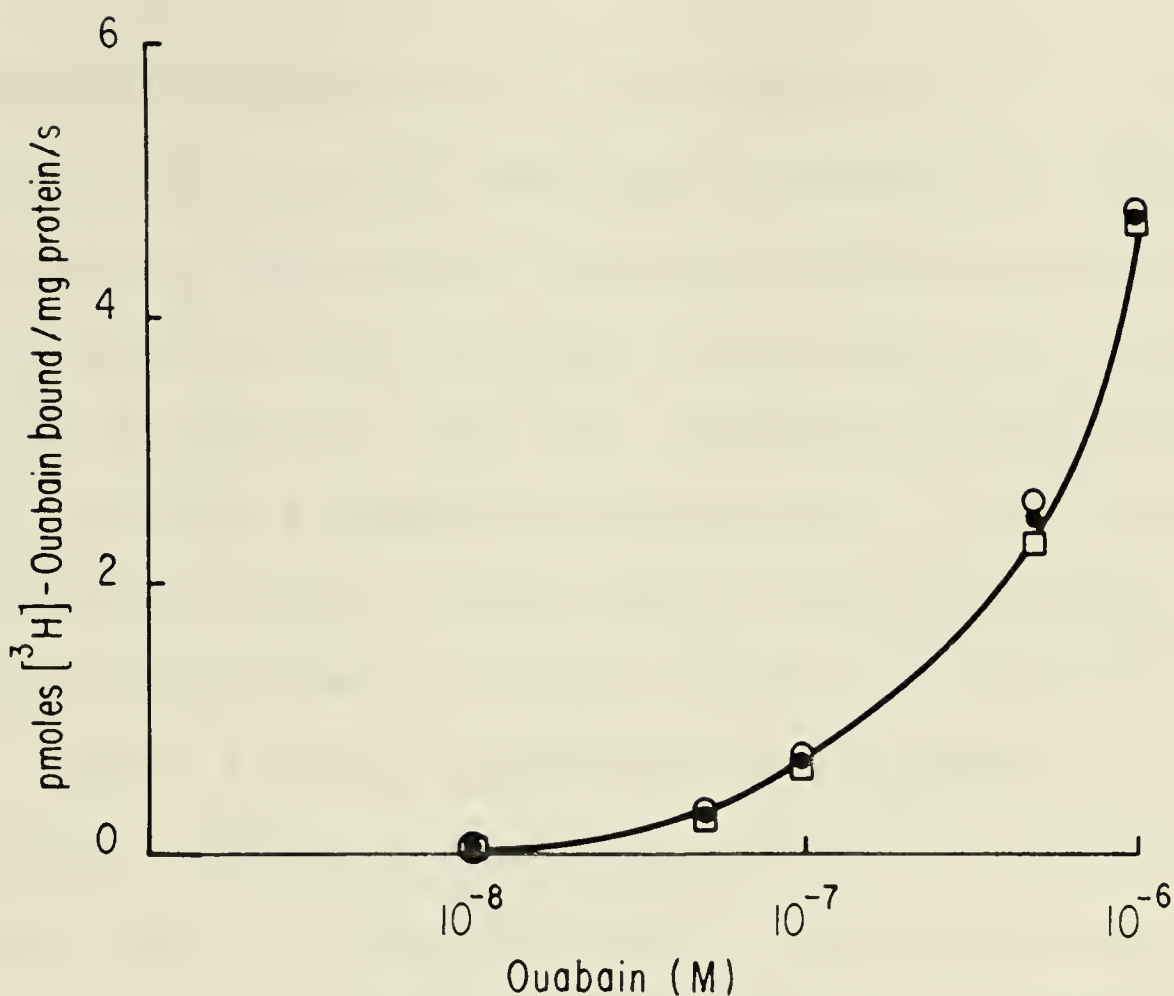


Fig. 7 Rate of [ $^3\text{H}$ ]-ouabain binding to a membrane preparation of ( $\text{Na}^+ + \text{K}^+$ )-ATPase at  $37^\circ\text{C}$ . The enzyme preparation had been extracted with 0.1% DOC at  $4^\circ\text{C}$  for 10 min. The specific activity of the preparation was  $23 \mu\text{moles Pi/mg protein/h}$ ; 80% of the total ATPase activity of the preparation was inhibited by 0.4 mM ouabain. Protein concentrations were adjusted to (●),  $90 \mu\text{g/ml}$ ; (□),  $180 \mu\text{g/ml}$  and (○),  $240 \mu\text{g/ml}$ . Results are given as pmoles ouabain bound/mg protein/s and are the means of duplicate determinations. Pre- and post- wash solutions contained unlabelled ouabain concentrations equal to the [ $^3\text{H}$ ]-ouabain concentration in the incubation medium as described under Methods.



first measurement was taken within 5 s of the addition of the enzyme protein. Therefore for technical reasons the drug concentration necessary for maximum rate of drug-receptor binding could not be determined by this method, despite the fact that five samplings were made within 30 seconds of initiating the reaction. Thus it was important to determine the effect of using possibly a submaximal concentration of  $5 \times 10^{-7}$  M ouabain for our drug binding study. This was done by determining binding rates for different concentrations of protein, and the results are also shown in Fig. 7. Despite a three fold variation in the concentration of the receptor protein, there was no significant difference in the rate of drug binding. That is to say, with respect to the abscissa there is no alteration in the slope of the line and this absence of any shift, would suggest that  $5 \times 10^{-7}$  M ouabain must be close to the maximum concentration required. In this regard it is of interest that a recent paper by Akera and Cheung (1977) describes a method of studying receptor binding reactions which is thought to overcome this problem. Using their methodology on our experiments, Simonson and Charnock (personal communication) have shown that  $5 \times 10^{-7}$  M ouabain yields a maximum value for ouabain binding which is about 5% greater than the extrapolated maximum calculated from the type of probit analysis used by Akera and Cheung (1977).

A second argument favouring  $5 \times 10^{-7}$  M ouabain as an appropriate concentration for drug binding studies can be developed as follows. At lower temperatures the drug binding rate would be slower than at the high temperatures and consequently a drug concentration of  $5 \times 10^{-7}$  M would yield maximal rates. Therefore a complete temperature study from 37°C to about 9°C would make use of maximal initial rates at most tem-



perature points and submaximal rates at the higher temperature points. In contrast, if a higher drug concentration was used, say  $5 \times 10^{-6} \text{M}$ , then maximal rates at the lower temperatures would remain unchanged while at the higher temperature points there would arise the possibility that equilibrium binding values would result. It was decided that a temperature study employing a combination of maximal and almost maximal initial rates was preferable to one employing a combination of maximal initial rates and equilibrium binding values. Thus like Erdmann and Schoner (1973a, 1974) drug binding was studied by using  $5 \times 10^{-7} \text{M}$  ouabain, which although not maximal at  $37^\circ\text{C}$  was technically convenient.

### 2.3 Drug binding and specific activity<sup>1</sup>

It is generally undisputed that inhibition of the enzyme occurs by the binding of ouabain to the catalytic subunit and the evidence for this belief was documented in the introduction. From this it follows that some relationship should exist between specific activity ( $\mu\text{moles Pi}$  liberated from ATP/mg total protein/hour) of the enzyme and its binding to ouabain. This relationship was investigated using ox brain ( $\text{Na}^+ + \text{K}^+$ )-ATPase enzyme preparations of widely variable specific activity obtained from both untreated and detergent treated preparations. Fig. 8 shows the results of more than 40 experiments, in which binding to these membranes was determined at  $37^\circ\text{C}$ . Initial rates were determined during the first 30 s of binding and these are shown in panel A, while equilibrium levels of drug binding which were determined after 10 min are shown in panel B. It is clear that the rate of ouabain binding increases with increasing specific activity of these preparations. The

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<sup>1</sup>Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) *Biochim. Biophys. Acta* 465, 77-92, (see appendix VI).





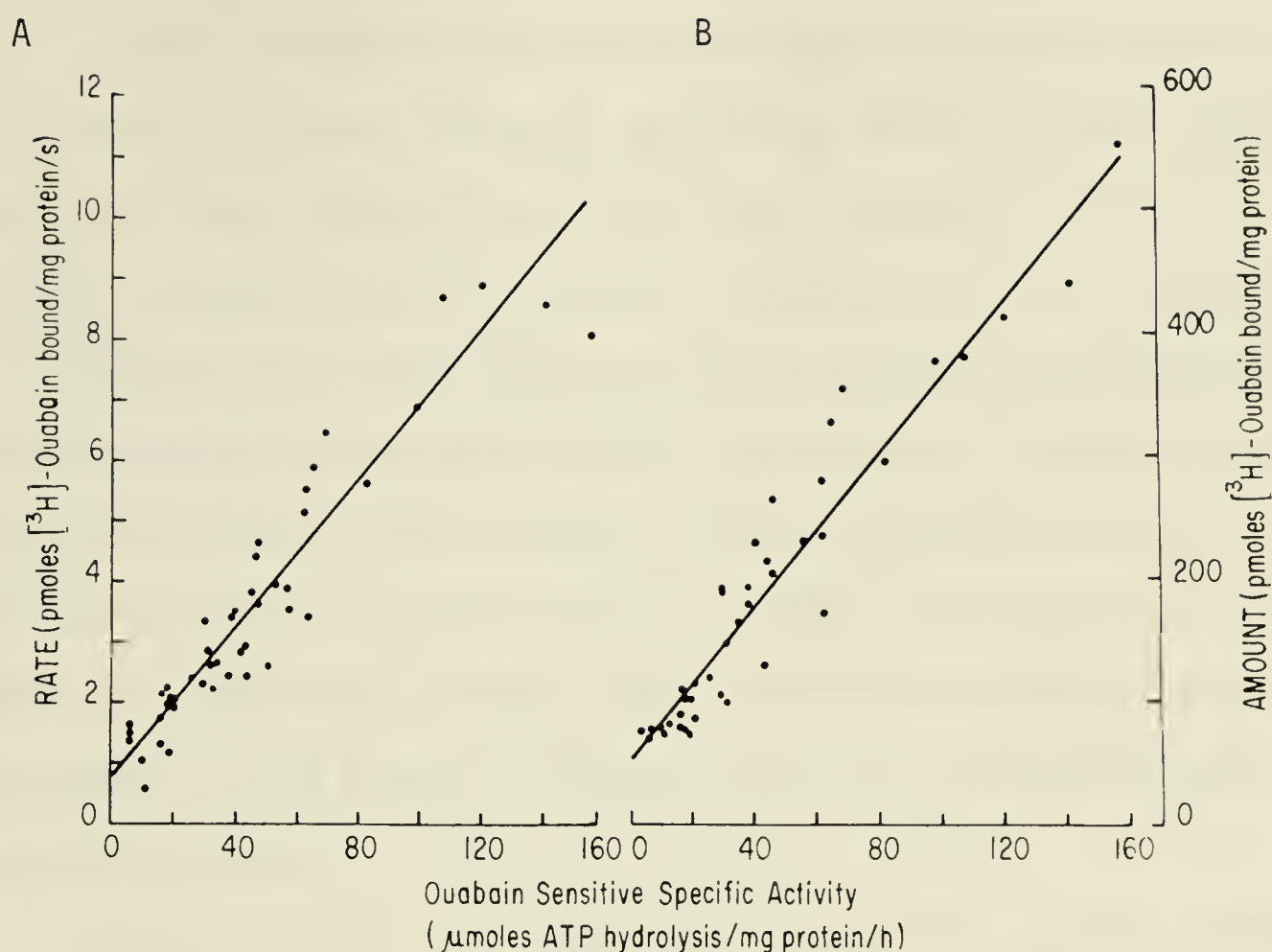


Fig. 8 Relationship of the specific activity of ouabain sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase with the rate of [ $^3\text{H}$ ]-ouabain binding (Panel A) and the amount of [ $^3\text{H}$ ]-ouabain bound at equilibrium (Panel B). All experiments were at 37°C. Enzyme preparations of widely different specific activity were obtained from untreated preparations and following the various detergent extractions with DOC and SDS described under Methods. Rates were determined from aliquots taken during the first 30 s after the addition of the enzyme. Each point represents a single enzyme preparation, assays were in duplicate or triplicate. Amounts of [ $^3\text{H}$ ]-ouabain binding at equilibrium were determined after 10 min incubation at 37°C and are the means of duplicate-assays.



correlation shown is significant at the 0.05 level or better. From panel B it is again evident that a good correlation is also obtained between the amount of ouabain bound at equilibrium at 37°C and the specific activity of the preparation. This latter correlation was also significant at the 0.05 level or better. These results are similar to those of Erdmann and Schoner (1973a, 1974) who suggested that binding studies conducted at equilibrium reflect the number of available ouabain binding sites per unit mass of enzyme, while rate studies reflect the affinity of these sites rather than their number. Thus the data in fig. 8 support the claim made by these authors that both the affinity as well as the number of drug binding sites show a positive correlation with the specific activity.

Despite this positive correlation between the specific activity of the enzyme and the two ouabain binding parameters (equilibrium level and rate of binding), a precise stoichiometric relationship remains to be determined. A major obstacle in its determination is the absence of a precise reference component. Because of this, the rate of ouabain binding has been re-examined with reference to the catalytic sites. Fig.9 ( shows a plot of the ratio of two rates namely ouabain binding ( $\mu\text{moles } [^3\text{H}]\text{-ouabain/mg protein/h}$ ) and specific activity ( $\mu\text{moles Pi/mg protein/h}$ ) versus the enzyme specific activity given as a rate of ATP hydrolysis. From this figure the following observations are made:

- a) The line is not parallel to the abscissa therefore the affinity of the receptor for ouabain, changes with the specific activity of the enzyme.
- b) The negative slope implies that the affinity of the receptor decreases as the enzyme is purified. Such a change in the



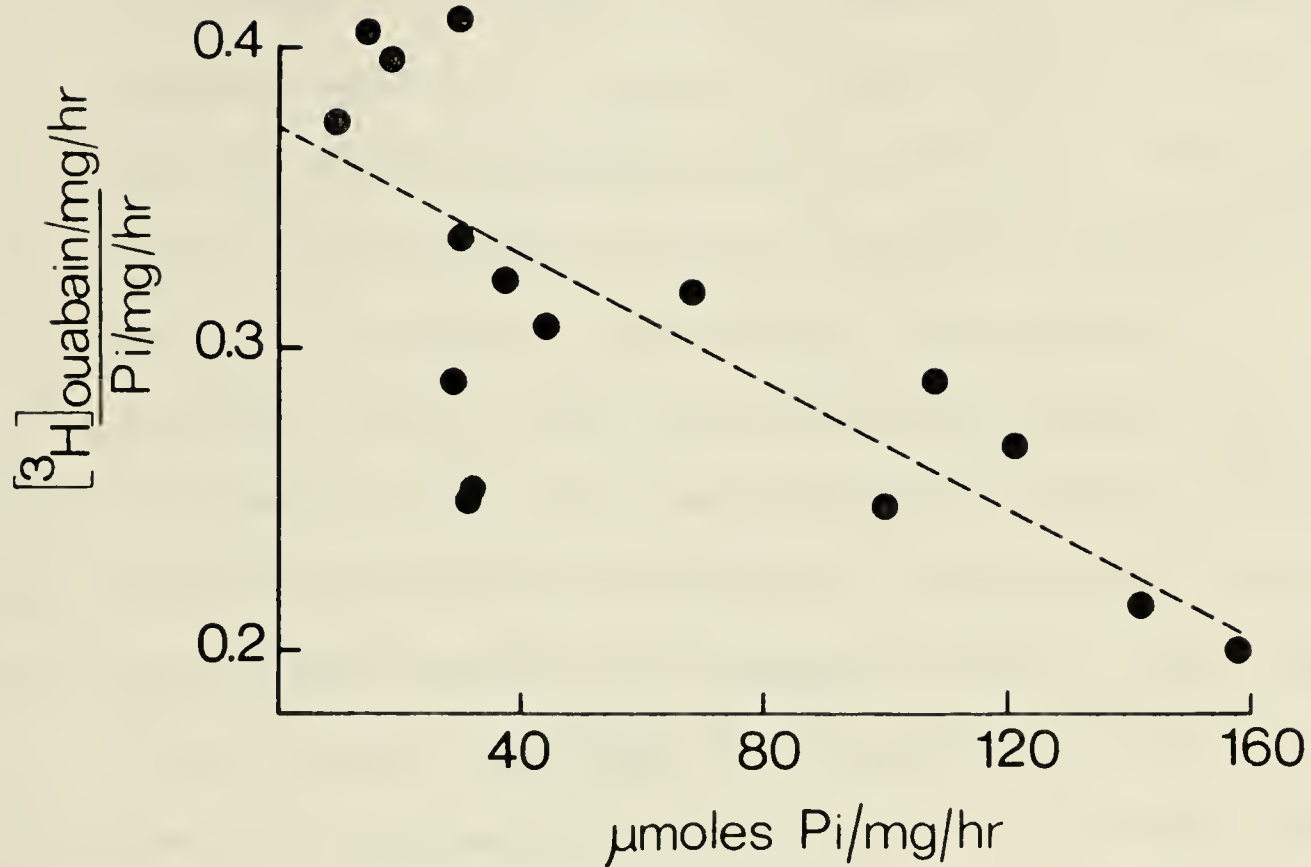


Fig. 9 The effect of specific activity ( $\mu\text{moles Pi/mg protein/hr}$ ) on the rate of binding of  $[^3\text{H}]$ -ouabain to ox brain microsomes. The rate of binding of ouabain is expressed as a measure of the specific activity.





receptor may be a consequence of detergent treatment but the data do not permit this degree of interpretation.

- c) The absence of a biphasic slope, composed of a horizontal line changing suddenly to a negative slope excludes the possibility that the drug concentration was rate limiting. This reinforces the interpretation that the negative slope of the line reflects a change in the affinity of the receptor.

Therefore from this data, the relationship between the affinity of the receptor for ouabain and the specific activity of the enzyme must at this point remain undefined. This question is discussed again along with other results in a subsequent section of this chapter.

Table 4 shows a comparison of the specific activity and equilibrium levels of bound ouabain bound to the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  after various treatments with detergent. It is apparent from the table that the specific activity can be gradually increased depending on the extent of the detergent treatment. Mild treatment with DOC at  $4^\circ$  leads to a relatively small increase in the specific activity of the enzyme while a more pronounced exposure to DOC or SDS at  $30^\circ$  in the presence of ATP produces marked increases in the specific activity. When the cations  $\text{Na}^+$  and  $\text{Mg}^{++}$  are present, the purification that results is intermediate to these two extremes. In correspondence with the gradual increase in catalytic sites there is also a gradual increase in the ouabain binding sites following detergent treatment. If however the two parameters are expressed as a ratio then a very different pattern results. It now appears that the drug binding sites decrease rather than increase with the catalytic sites. It is noted however that two of the five treatments yield similar ratios (4.6 and 4.8)



TABLE 4

## COMPARISON OF THE SPECIFIC ACTIVITY AND THE EQUILIBRIUM BINDING OF OUABAIN

Treatment	n	Specific <sup>1</sup> activity	Equilibrium <sup>2</sup> binding	EB/SA
None	14	15.2 ± 2.5	90.5 ± 6.1	5.95
DOC 4°	16	31.6 ± 3.2	146.5 ± 12.7	4.64
DOC + ATP + Na + Mg at 30°	7	60.9 ± 4.8	295. ± 26	4.84
DOC + ATP at 30°	3	96.9 ± 18.0	342. ± 55	3.53
SDS + ATP at 30°	3	133.0 ± 17.0	457. ± 51	3.44

- 1  $\mu$ moles Pi/mg protein/hr.\*
2. pmoles [<sup>3</sup>H]-ouabain/mg protein.\*
- \* ± standard error of the mean.



despite the fact that the specific activity of these two preparations showed a two-fold difference. From this data it is not possible to say which of these two treatments is incongruous to the series. Nevertheless the study on drug binding plus the influence of specific activity poses two questions of considerable importance to this project.

- a) What molecular mechanism is responsible for the change in the number of ouabain binding sites and possibly their affinity as the enzyme is purified.
- b) Does the change in rate of ouabain binding with purification offer a clue to the toxicity of this drug.

### 3. Ouabain binding and temperature<sup>1</sup>

Earlier work from this laboratory with which I was involved, (see appendix II) had shown that enzyme inhibition by ouabain was influenced to a greater extent by change in the temperature than was the activation of the enzyme by cations. However it was not possible from that work to assess whether the initial rate of ouabain binding or the equilibrium level of drug binding was involved. Thus in pursuing this aspect, the effect of temperature on both these parameters of drug binding was first examined in detail.

#### 3.1 The effects of temperature on the rate of ouabain binding<sup>1</sup>.

The effect of temperature upon the rate of ouabain binding to untreated preparations of ox brain enzyme can be seen from the results shown in Fig. 10. As would be expected the rate of drug

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<sup>1</sup> Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) *Biochim. Biophys. Acta* 465, 77-92. (See appendix VI).





Fig. 10 The effect of temperature on the binding of [ $^3\text{H}$ ]-ouabain to an untreated membrane preparation of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . The enzyme preparation had a specific activity of  $16.5 \mu\text{moles Pi/mg protein/h}$ ; 82% of the total ATPase activity of the preparation was inhibited by  $0.4\text{mM}$  ouabain. The protein concentration was  $107 \mu\text{g/ml}$ . The rate of [ $^3\text{H}$ ]-ouabain binding was determined from the slope of the line obtained at each experimental temperature, using the Olivetti programme #681009 as described under Methods. The mean initial rates determined in this experiment were from  $9.5^\circ\text{C}$  -  $36.9^\circ\text{C}$  respectively  $0.05, 0.08, 0.13, 0.20, 0.59, 0.89, 1.24$  and  $1.95 \text{ pmoles } [^3\text{H}]\text{-ouabain/mg protein/s}$ . Standard error of the means of each temperature were always  $<10\%$ . Assays were in duplicate or triplicate.



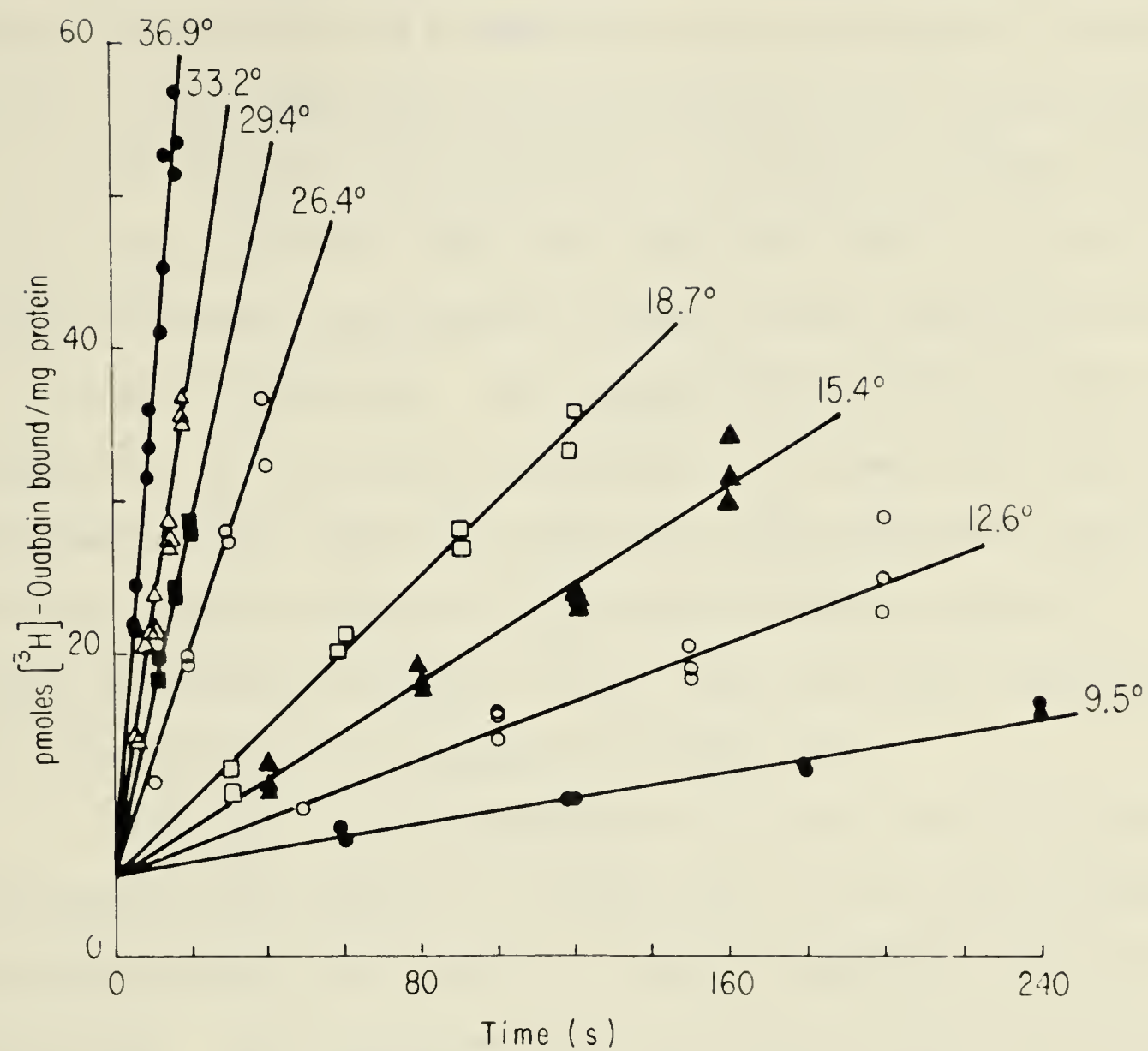


Fig. 10.

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binding decreases as the temperature is lowered. It is also noteworthy that under the conditions of our experiments the rate of binding was linear at all the 8 temperatures that were examined from 36.9° to 9.5°. This supports our earlier observation that a concentration of  $5 \times 10^{-7}$  M ouabain, was suitable for our studies. The mean initial rates were 0.05, 0.08, 0.13, 0.20, 0.59, 0.89, 1.24 and 1.95 pmoles [ $^3\text{H}$ ]-ouabain /mg protein/s for the different temperatures from 9.5° to 36.9° respectively. The standard errors of the means for the binding rate at the different temperatures were always less than 10%. Experiments such as these were conducted in many subsequent investigations to study the effects of temperature on membranes exposed to different delipidation treatments. In all cases mean rates obtained in this manner were used to construct Arrhenius plots.

From fig. 10, the observation is also made that the linear time period of drug uptake i.e. binding times varied at the different temperatures being less than 30 s at higher temperatures and upto 4 min at 9.5°. The time periods were held to these values in all subsequent experiments.

### 3.2 The effects of temperature on the equilibrium binding of ouabain.<sup>1</sup>

The effect of temperature on the amount of [ $^3\text{H}$ ]-ouabain which was bound to membrane ( $\text{Na}^+ + \text{K}^+$ )-ATPase preparations at equilibrium was also examined. The results of a study using untreated enzyme preparations is given in table 5. Since the rate of [ $^3\text{H}$ ]-ouabain binding de-

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<sup>1</sup> Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) *Biochim. Biophys. Acta* 465, 77-92, (See appendix VI).





TABLE 5

EQUILIBRIUM LEVELS OF OUABAIN BOUND TO BEEF BRAIN(Na<sup>+</sup> + K<sup>+</sup>)-ATPase AT VARIOUS TEMPERATURES

Temperature (°C)	[ <sup>3</sup> H]-ouabain bound pmole/mg protein ± S.D.		
36.8	87	±	5.7
33	90.6	±	2.3
29.3	85	±	2.5
26.5	94.6	±	5.2
18.5	94.3	±	5.2
15.3	89	±	3.6
12	85	±	4.8
9.3	93.2	±	4.2*

In this series of experiments the binding reactions were started by the addition of protein to a final concentration of 225 µg/ml and terminated by filtration after 30 min, except for the lowest temperature\* where equilibrium was not reached until 60 min after protein addition. The concentration of ouabain was  $5 \times 10^{-7}$  M in all experiments. The values given are the means ± S.D. of at least 4 experiments at each temperature.



creases with temperature, the binding reaction was allowed to proceed for 30 min in all cases except at the lowest temperature where equilibrium was not reached until 60 min after the reaction had been initiated. The latter was achieved by the addition of membrane protein to a final concentration of 225 µg/ml. The results clearly show that at true (demonstrated) equilibrium, temperature does not affect the amount of drug which can be bound; that is, a variation in the experimental temperature does not alter the number of ouabain receptors available for binding.

### 3.3 The effects of phospholipase-A on ox brain membranes<sup>1</sup>.

The earlier work with rabbit kidney enzyme had shown that phospholipase-A had a marked effect on the specific activity of the enzyme and its sensitivity to temperature. In addition, from the results presented so far it is clear that purification of the enzyme from ox brain, influences the affinity as well as the number of ouabain receptors. In this regard, Taniguchi and Iida (1971, 1973) have reported that treatment of the enzyme with PPL-A resulted in a loss of activity and a reduction in the initial rate of [<sup>3</sup>H]-ouabain binding. These workers also reported that the binding capacities of the ouabain binding site showed no remarkable change as a consequence of treatment with phospholipase-A. Thus, this lipase plays an important role in the investigation of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase, and this section discusses these effects in greater detail.

In this examination it was found that the effects of PPL-A varied dependent upon the degree of lipolysis which was obtained and

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<sup>1</sup>Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) Biochim.Biophys.Acta 465, 77-92 (See appendix VI).



also on the pretreatment of the enzyme with detergent. These two aspects are discussed separately.

Fig. 11 shows the effects of PPL-A on membranes prepared by Polytron homogenization (method (b), p. 36). In these experiments lipolysis was achieved by exposure of the membrane to 20 units bee venom PPL-A for a period of 40 minutes. Samples were removed at intervals and the membrane enzyme was recovered. Under these conditions, untreated ox brain membranes undergo a biphasic effect. Over the first 5 minutes there is an increase in specific activity of the enzyme, followed by a progressive fall such that within 15 minutes the level has returned to that of the pretreated membranes, and remains unchanged for the remainder of the treatment period. In the case of the ouabain binding there is a corresponding increase in the level at about 5 minutes followed by a fall which remains at a steady state after about 15 minutes. It is notable however, that whereas the specific activity returned to the pretreatment level, the amount of ouabain that was bound at the steady state level was greater than the pretreatment level by about 50%. Thus in contrast to the results of Taniguchi and Iida (1971, 1973) that were cited above, this work clearly shows that lipolysis with PPL-A does not result in a marked reduction in the amount of ouabain that is bound to the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ .

If the membranes were first extracted with DOC at 4°C, the biphasic effect of PPL-A was lost. This is shown in Fig. 12. After treatment with this detergent the "initial" levels of both ouabain sensitive  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity as well as  $[^3\text{H}]$ -ouabain binding are much higher than those of untreated enzyme preparations observed previously. Now the action of PPL-A is to produce an immediate and pro-





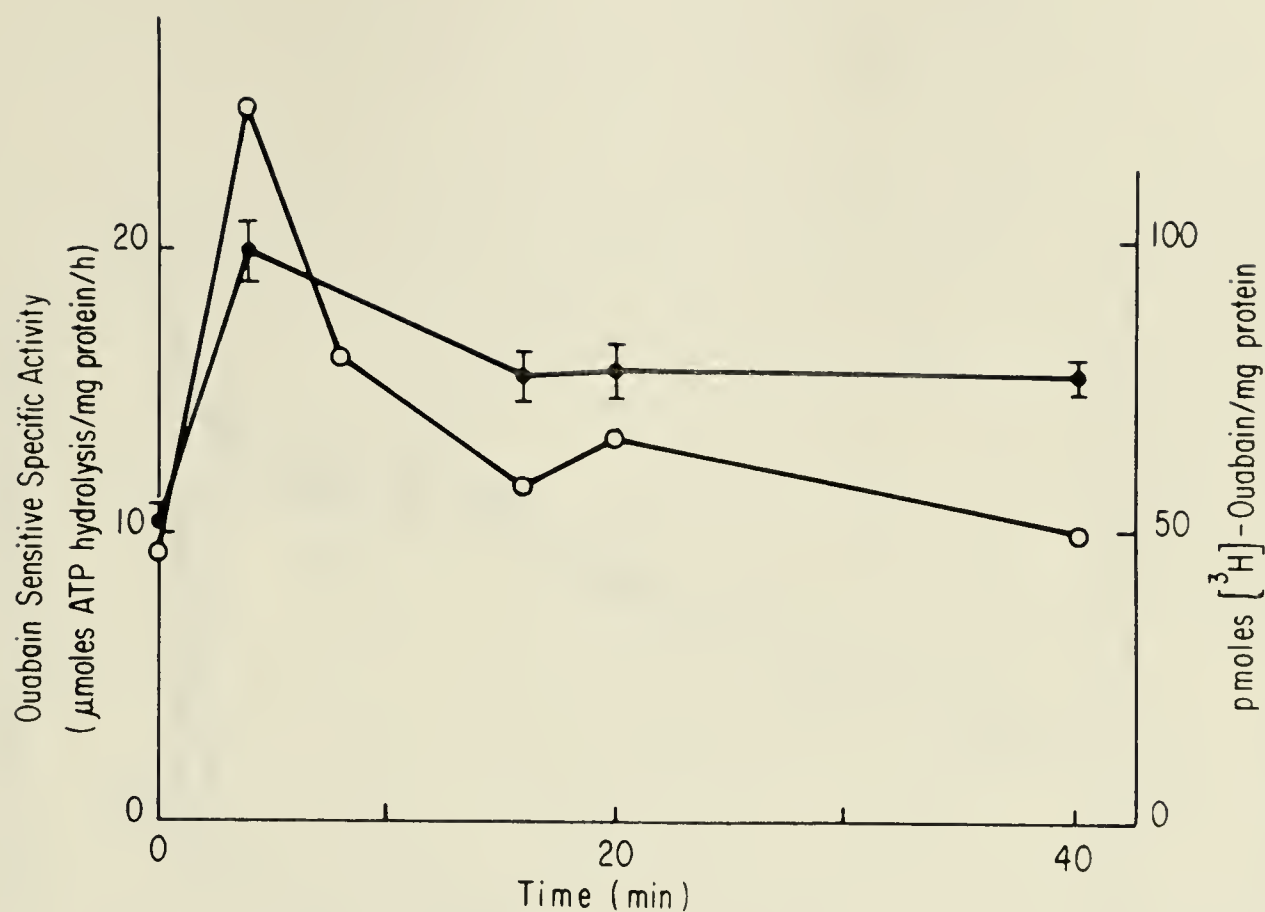


Fig. 11 Effect of progressive treatment at 37°C with 20 units of bee venom phospholipase-A/mg protein on the specific activity and binding of [<sup>3</sup>H]-ouabain at equilibrium to otherwise untreated membrane preparations of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase. (●), [<sup>3</sup>H]-ouabain binding given as pmoles/mg protein, values are the means  $\pm$  S.E. of triplicate determinations from 1 ml aliquots taken after 5 min incubation at 37°C. (o), specific activity given as μmoles Pi/mg protein/h at 37°C, values are means of duplicate assays.



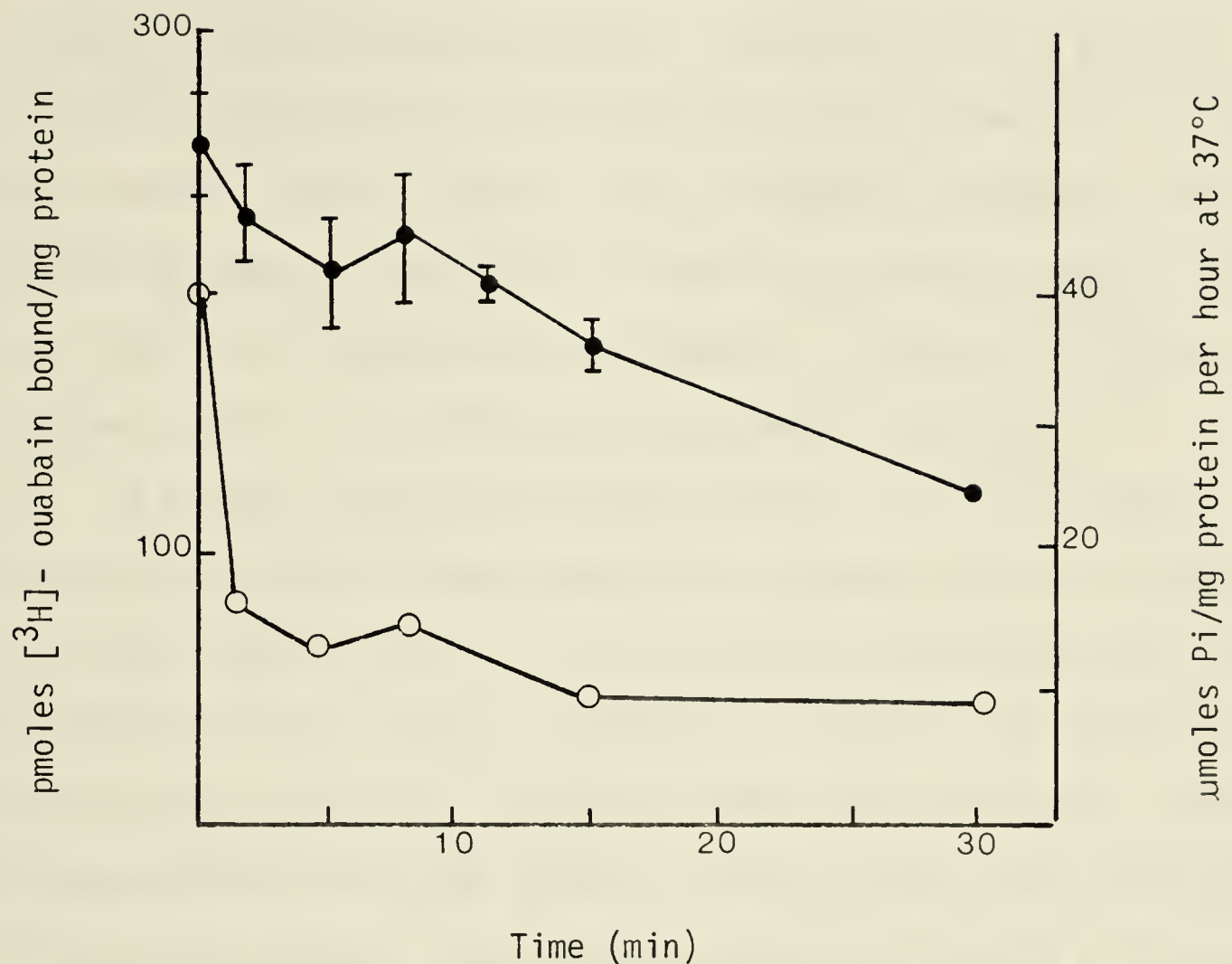


Fig. 12. Effect of progressive treatment at  $37^\circ\text{C}$  with 5 units of bee venom phospholipase-A<sub>2</sub>/mg enzyme protein on the specific activity and binding of  $[^3\text{H}]\text{-ouabain}$  at equilibrium to membrane preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  after 10 minutes extraction with 0.1% deoxycholic acid at  $4^\circ\text{C}$ . (●),  $[^3\text{H}]\text{-ouabain}$  binding given as pmol/mg protein, values are means  $\pm$  S.E. of four times 1-ml aliquots taken after 5 minutes incubation at  $37^\circ\text{C}$ ; (○), specific activity given as  $\mu\text{mole Pi/mg protein per h at } 37^\circ\text{C}$ ; values are means of duplicate assays.



gressive decline in the specific activity of the preparations. It is interesting that the lipase resistant level of the catalytic activity is about the same as that seen with the untreated enzyme preparation, i.e. about 10  $\mu$ moles Pi/mg protein/hr. The sensitivity to PPL-A of the ouabain binding sites in these detergent treated membranes is not quite so marked. As the exposure time to lipase is increased, there is a gradual decrease in the amount of ouabain that becomes bound. Thus, as was observed with the untreated membranes, the amount of ouabain bound does not fall in parallel with the specific activity.

A further illustration of this effect is shown in table 6. Here the enzyme which had been prepared by homogenization in a glass mortar with a teflon pestle (method 'a' p. 36) was first extracted with 0.1% DOC at 4°C for 10 min and then subjected to lipolysis with 5 units of phospholipase-A for 5 min. A total of seven preparations were examined for enzyme specific activity as well as the rate and equilibrium level of [ $^3$ H]-ouabain binding. From the data presented in table 6 it can be seen that the three parameters mentioned above all undergo a change. The activity of the enzyme is most sensitive to lipolysis and is reduced to about 15% of the control value, while the rate and the equilibrium binding of ouabain are reduced to 53% and 73% respectively. That is, both the biochemical activity of the enzyme and the affinity of the binding sites are more susceptible to lipolysis with phospholipase-A than are the number of drug receptors.

#### 3.4 Arrhenius plots of [ $^3$ H]-ouabain binding to untreated ox brain membranes<sup>1</sup>.

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<sup>1</sup>Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) *Biochim.Biophys.Acta* 465, 77-92 (See appendix VI).





TABLE 6

COMPARISON OF THE EFFECTS OF PHOSPHOLIPASE-A ON THE SPECIFIC  
ACTIVITY OF, AND THE RATE AND EQUILIBRIUM LEVELS OF [ $^3\text{H}$ ]-OUABAIN  
BINDING TO  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}^1$

	n	S.A. <sup>2</sup>	<sup>3</sup> Rate of [ $^3\text{H}$ ]- ouabain bound	Equilibrium <sup>4</sup> level of [ $^3\text{H}$ ]- ouabain bound
Control	7	26.3 $\pm$ 4.3	2.70 $\pm$ 0.29	137 $\pm$ 23
After phospholipase-A	7	3.8 $\pm$ 0.9	1.42 $\pm$ 0.31	100 $\pm$ 18
Difference%		85.4	47.4	27.0

<sup>1</sup>Enzyme was treated with 0.1% DOC at 4°C for 10 min following pre-  
paration by method 'a' as described on p. 36.

<sup>2</sup>Mean specific activity in  $\mu\text{moles Pi/mg protein/hr} \pm \text{S.E.}$

<sup>3</sup>Mean pmoles [ $^3\text{H}$ ]-ouabain bound/mg protein/s  $\pm$  S.E.

<sup>4</sup>Mean pmoles [ $^3\text{H}$ ]-ouabain bound/mg protein  $\pm$  S.E.



As was described in the chapter on methods two procedures were employed for the homogenization of ox brain, and both membrane preparations were examined for the effects of temperature on the initial rate of ouabain binding. The results, presented as Arrhenius plots are shown in fig. 13. From the mean data obtained from five separate experiments using untreated beef brain membranes prepared by limited teflon-glass homogenization (method a), it was possible to construct an Arrhenius plot for the binding of [ $^3\text{H}$ ]-ouabain. This plot is clearly non-linear, and can be described by two straight lines intersecting at a transition temperature ( $T_c$ ) of about  $25^\circ\text{C}$ . Calculation of the apparent energies of activation above ( $E_{a_1}$ ) and below ( $E_{a_2}$ ) the critical temperature, yields values of  $18.5 \pm 2.1$  and  $29.1 \pm 1.3$  kcal/mole respectively. Comparison of these mean values by the paired "t" test indicates significance at the 0.01 level.

The mean data from another group of six experiments using untreated beef brain enzymes prepared by disintegration with the Polytron, (method b) have also been shown as an Arrhenius plot in fig. 13. Once again the plot is non-linear with a similar value for the transition temperature of about  $25^\circ\text{C}$ . The mean values for the activation energies  $E_{a_1}$  and  $E_{a_2}$ , derived from this plot are  $20.9 \pm 0.33$  and  $25.7 \pm 0.86$  kcal/mole respectively. It is notable that these values for  $E_{a_1}$  and  $E_{a_2}$  are not as widely divergent as was seen with the untreated membranes obtained by glass-teflon homogenization (method 2 above). Comparison of the two slopes by the paired "t" test demonstrated statistical significance at the 0.001 level.

Non-linear Arrhenius plots obtained with data generated by membrane bound enzymes are generally interpreted as phase transitions or



Fig. 13. Arrhenius plots of the rate of [ $^3\text{H}$ ]-ouabain binding to ( $\text{Na}^+ + \text{K}^+$ )-ATPase from ox brain prepared by method 'a' ( $\blacktriangle$ ) and by method 'b' ( $\circ$ ). For comparison the rates obtained at  $37^\circ\text{C}$  were set at 100% and the experimental values obtained at other temperatures were expressed as a percentage of this control. To clarify presentation in this form the data from membranes prepared by method 'a' have been offset. The results shown are the means obtained from not less than three experiments.





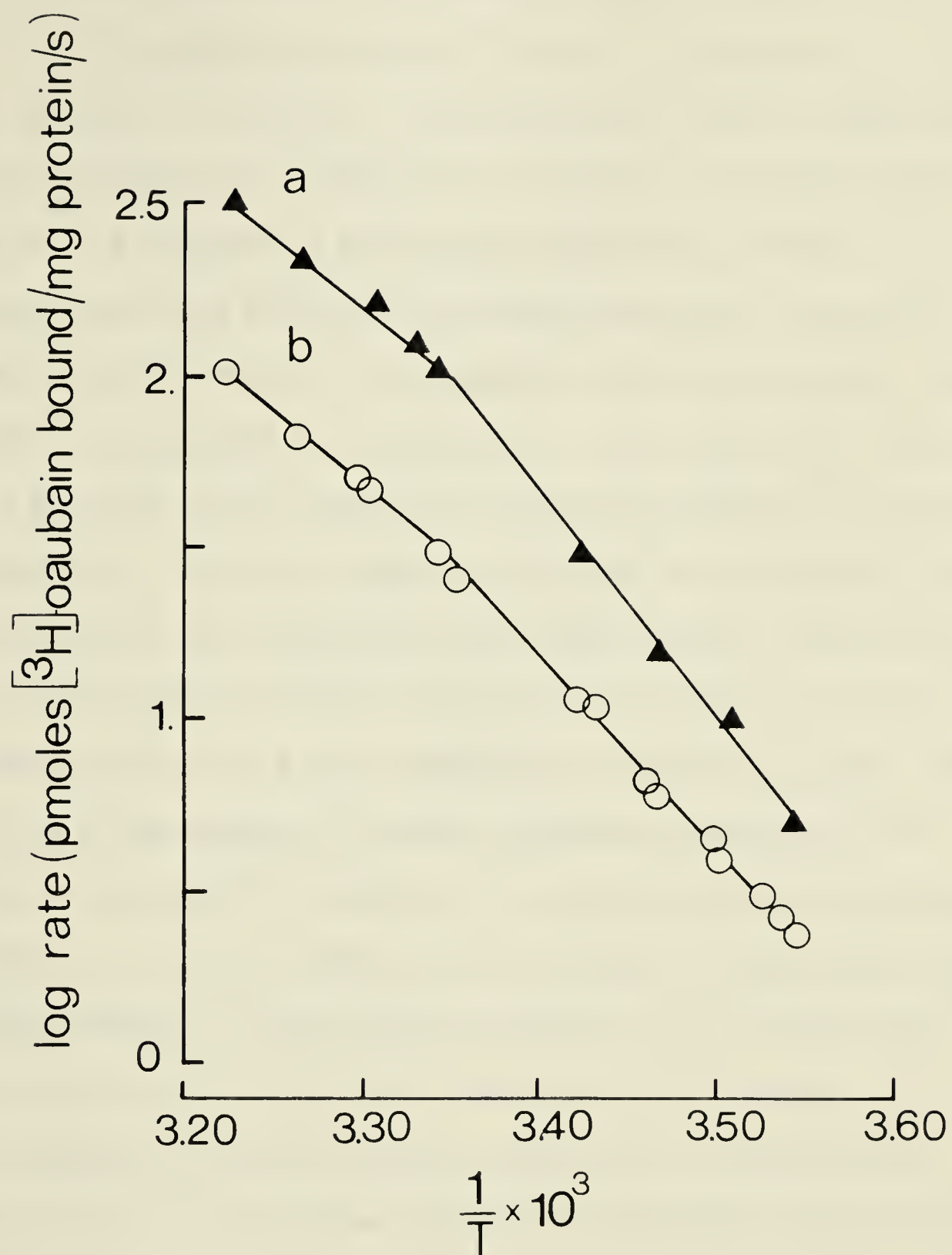


Fig. 13. Legend on separate page.



changes in the lipid bilayer. By analogy it can be inferred from Fig. 13 that the membrane lipids are involved in the binding of [ $^3\text{H}$ ]-ouabain to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . This supports our earlier suggestion, (Charnock, Almeida and To, 1975) that the cardiac glycoside sensitivity of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  is a phenomenon mediated by lipids. It is nevertheless puzzling that the two untreated membrane preparations are not identical with respect to the apparent activation energies. When the latter are expressed as a ratio of  $E_{a1} : E_{a2}$ , the values 0.64 and 0.81 are obtained for the enzymes as prepared by method [a] and method [b] respectively. Since the membranes obtained from method [b] generate an Arrhenius plot that more closely approximates a single straight line, it would appear that with respect to the binding of ouabain, these membranes have lost their temperature sensitivity to some degree. This may be a consequence of the more vigorous disruption of the membranes by the polytron, leading to a preparation with altered residual lipids. If such an alteration is possible, it also implies that the lipids involved in the binding of ouabain to the protein macromolecule can be at least partially removed by this treatment. A second difference in the two enzyme preparations is the increased specific activity of the enzyme prepared by method [b] (see appendix IV). This difference of 5  $\mu\text{moles Pi/mg protein/hr}$  (15 and 20 respectively for the glass-teflon and polytron preparations) is too small to convincingly implicate the specific activity in the altered temperature sensitivity of the ouabain binding reaction, but not small enough to be ignored.



### 3.5 Arrhenius plots of [ $^3\text{H}$ ]-ouabain binding to detergent treated ox brain membranes<sup>1</sup>.

In a previous section (1.3) data were presented which showed that the temperature sensitivity of the catalytic activity of ox brain ( $\text{Na}^+ + \text{K}^+$ )-ATPase, varied consequent to the method of preparation. In particular, the enzyme preparation obtained after polytron homogenization, method [b] yielded activation energies,  $E_{a1}$  and  $E_{a2}$  that were smaller by about 20%; in this respect they bore some similarity to the detergent treated membranes. This lack of correlation between membranes prepared by method [a] and by method [b] was also reflected in the [ $^3\text{H}$ ]-ouabain binding studies presented in the section (3.4) immediately above. Thus a similarity exists between the temperature sensitivity of the catalytic activity on the one hand and the binding of ouabain on the other. In order to further examine the effects of temperature on the binding of ouabain, detergent treated membranes were studied.

Fig. 14 shows the Arrhenius plots obtained from a series of experiments which measured the binding of ouabain to membranes that had been treated with DOC under various conditions. If mild detergent extraction is employed, 0.1% DOC at 4°C for 10 min, it reveals differences in the enzyme-enriched membranes obtained by the two different preparative procedures. With enzyme preparations from method [a], there are some obvious quantitative changes following extraction with DOC. The thermal transition in the Arrhenius plot of [ $^3\text{H}$ ]-ouabain bound is still clearly apparent, although the mean transition is now somewhat higher

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<sup>1</sup>Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) *Biochim.Biophys.Acta* 465, 77-92, (See appendix VI).





than before, at  $29^{\circ}\text{C} \pm 1.3^{\circ}\text{C}$ . There is also an alteration in the values of  $E_{a_1}$  ( $11.7 \pm 1.3$  kcal/mole) and  $E_{a_2}$  ( $21.8 \pm 2.2$  kcal/mole). Nevertheless the non-linearity of ouabain binding to the untreated membrane has remained obvious.

If however enzyme preparations obtained from method [b] were similarly treated and examined the results were quite different. The mean value for  $E_{a_1}$  at  $20.1 \pm 1.35$  kcal/mole was no longer significantly different ( $p > 0.4$ ) from the mean value for  $E_{a_2} = 22.1 \pm 1.59$  kcal/mole. That is, this form of mild detergent extraction of these beef brain preparations has given a product which no longer clearly displays a marked thermal transition for  $[^3\text{H}]$ -ouabain binding. Preparations obtained by the presumably less disruptive method [a] are more resistant to alteration by this form of detergent treatment than are preparations obtained by method [b].

In addition Fig. 14 also shows the results obtained by further treatment of beef brain enzymes (prepared by method [b]) with DOC at higher temperatures. All these more vigorous procedures also yield data which can best be described by linear Arrhenius plots; the computer assisted Bogartz analysis described in the section on methods does not reveal either statistically significant differences in the activation energies which were calculated for  $E_{a_1}$  and  $E_{a_2}$ , nor does it yield values for the transition temperatures which fall within the experimental range examined. This linearity of the temperature dependence of the rate of  $[^3\text{H}]$ -ouabain binding can be more easily seen if a complete set of numerical data are presented in tabular form.

Thus table 7 presents numerical data obtained from a variety of ouabain binding studies. In addition to those treatments already



Fig. 14. Arrhenius plots of the rate of [ $^3\text{H}$ ]-ouabain binding to ( $\text{Na}^+$  +  $\text{K}^+$ )-ATPase after various treatments. For comparison the rates obtained at  $37^\circ\text{C}$  were set at 100% and the experimental values obtained at other temperatures were expressed as a percentage of this control. To clarify presentation in this form the data from the different treatments were arbitrarily offset. Ox brain ( $\text{Na}^+$  +  $\text{K}^+$ )-ATPase prepared by method 'a' after treatment with 0.1% DOC at  $4^\circ\text{C}$  for 10 min ( $\blacktriangle$ ). Ox brain ( $\text{Na}^+$  +  $\text{K}^+$ )-ATPase prepared by method 'b' after treatment with 0.1% DOC at  $4^\circ\text{C}$  for 10 min ( $\blacksquare$ ); 0.05% DOC + 2mM ATP + 5mM  $\text{MgSO}_4$  + 80mM NaCl at  $30^\circ\text{C}$  for 5 min ( $\square$ ); 0.1% DOC + 3mM ATP at  $30^\circ\text{C}$  for 30 min ( $\circ$ ). Results shown are the means obtained from not less than three and sometimes seven experiments.



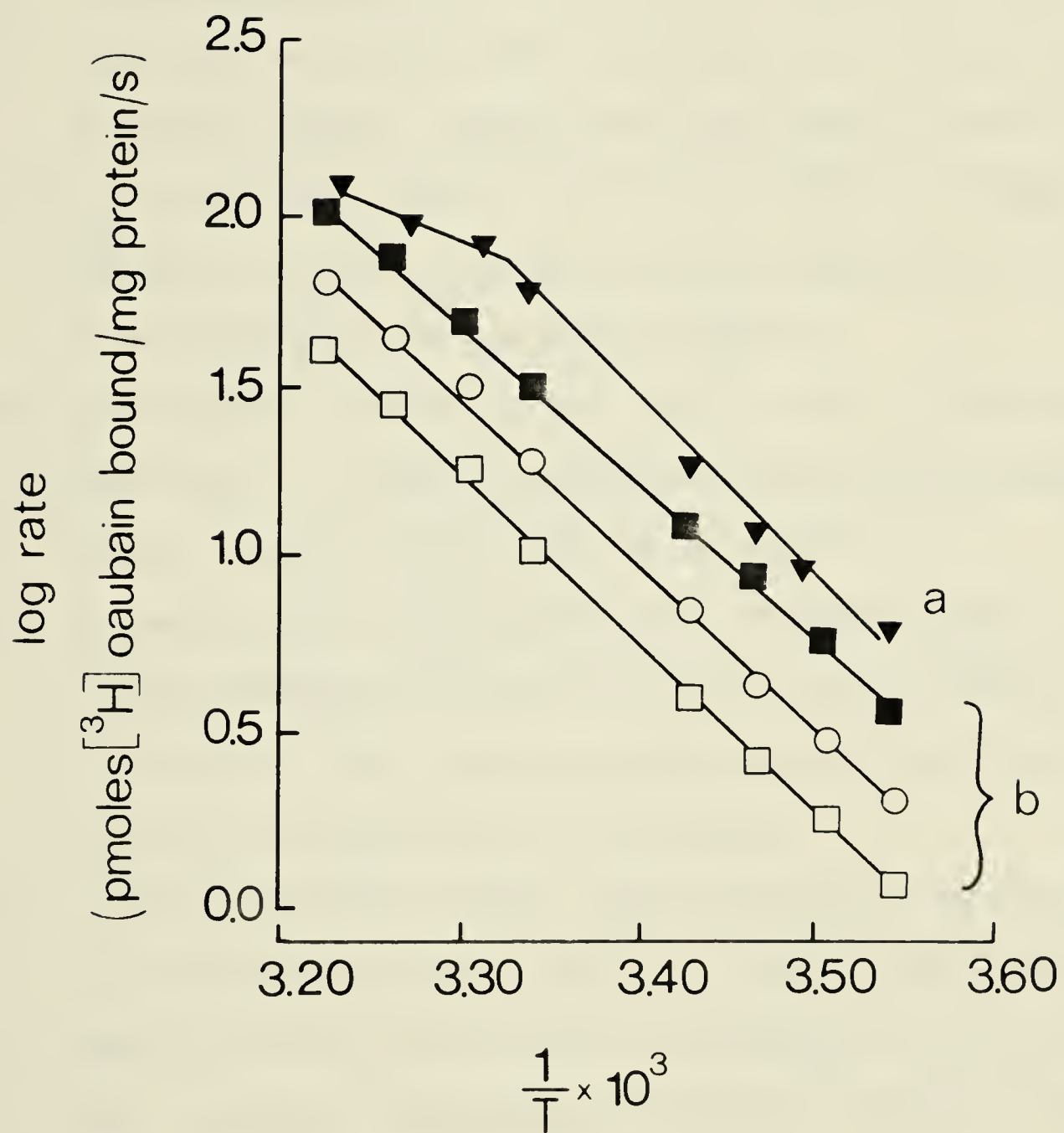


Fig. 14. Legend on separate page.





described in this section data are included from experiments carried out with membranes purified by extraction with SDS. A summary of the observations that can be made from Arrhenius plots of this [ $^3\text{H}$ ]-ouabain binding data is listed below:

- 1) Untreated membranes, irrespective of the method of preparation, yield non-linear plots. Furthermore the values of the  $T_c$  are also similar. However, the relationship of the  $E_{a_1}$  to  $E_{a_2}$  is noticeably different for the two methods of preparation. Membranes prepared by the more disruptive method (b), yield an Arrhenius plot that approaches linearity.
- 2) Mild detergent treatment alters the  $T_c$  as well as the activation energies of membranes prepared by Glass-teflon homogenization. However the non-linearity is retained.
- 3) In contrast, mild detergent treatment of the membranes obtained by homogenization with the polytron are no longer significantly non-linear, and the importance and reliability of the value calculated for  $T_c$  is decreased.
- 4) Treatment with DOC at higher temperatures and in the presence of ATP abolishes the non-linearity. If  $\text{Na}^+$  and  $\text{Mg}^{++}$  are present this result is not altered. Furthermore, a similar result is obtained with membranes purified by exposure to SDS at  $30^\circ\text{C}$  in the presence of ATP.
- 5) Treatment of the membranes with phospholipase-A also causes a loss of the characteristic non-linearity of Arrhenius plots of ouabain binding to untreated membranes. Unlike its effect upon specific activity the addition of PS to these partially



TABLE 7

APPARENT ACTIVATION ENERGIES CALCULATED FROM THE RATE OF [ $^3\text{H}$ ]-OUABAIN BINDING TO VARIOUS  
( $\text{Na}^+ + \text{K}^+$ )-ATPase PREPARATIONS AT DIFFERENT TEMPERATURES

#	Treatment	n	$E_{a1} \pm \text{SE}$ kcal/mole	$E_{a2} \pm \text{SE}$ kcal/mole	$\frac{E_{a1}}{E_{a2}}$	$T \pm \text{SE}$ $^{\circ}\text{C}$	P ( $E_{a1}, E_{a2}$ )
1	*Untreated	4	$18.5 \pm 2.10$	$29.1 \pm 1.30$	0.64	$25.4 \pm 2.00$	$<0.01$
2	Untreated	6	$20.9 \pm 0.33$	$25.7 \pm 0.86$	0.81	$24.6 \pm 1.40$	$<0.001$
3	*DOC - $4^{\circ}\text{C}$	7	$11.7 \pm 1.35$	$21.8 \pm 2.20$	0.54	$29.0 \pm 1.30$	$<0.01$
4	DOC - $4^{\circ}\text{C}$	3	$20.1 \pm 1.35$	$22.1 \pm 1.59$	0.91	$27.2 \pm 8.26$	$>0.40 \dagger$
5	DOC + ATP - $30^{\circ}\text{C}$	6	$20.4 \pm 0.56$	$21.9 \pm 0.37$	0.93	$43.7 \pm 3.05$	$>0.05 \dagger$
6	DOC + ATP + Mg + Na - $30^{\circ}\text{C}$	5	$22.8 \pm 0.37$	$21.5 \pm 1.79$	1.06	$9.5 \pm 2.25$	$>0.50 \dagger$
7	SDS + ATP - $30^{\circ}\text{C}$	3	$21.9 \pm 1.84$	$21.4 \pm 1.06$	1.02	$3.15 \pm 3.41$	$>0.80 \dagger$
8	**DOC - $4^{\circ}\text{C}$ + PPL-A	3	$26.5 \pm 2.43$	$25.8 \pm 2.31$	1.03	$-27.0 \pm 12.4$	$>0.80 \dagger$
	DOC - $4^{\circ}\text{C}$ + PPL-A + PS	2	$22.2 \pm 0.80$	$24.3 \pm 6.25$	0.91	$40.2 \pm 35.7$	$>0.70 \dagger$

\* Membranes prepared by glass-teflon homogenization, the remainder with the Polytron PT20.

\*\* 5 units PPL-A for 5 min at  $37^{\circ}\text{C}$ .

†Not significant.



delipidated preparations does not restore the non-linearity.

- c) Finally it can be concluded that the binding of ouabain to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  bears the characteristic thermal sensitivity that is common to its hydrolytic activity. In this case however the temperature effects can be relatively easily altered, as for example by homogenization with the Polytron PT 20, or by treatment with detergents.

#### 4. Spin labelled membranes

##### 4.1 Types of spectra.

Some of the characteristics of the spin probe M 12-NSE were described in the chapter on methods; three properties of this probe are pertinent to this section and are listed below:

- a) When examined spectroscopically a solution of this spin probe in methanol yields a typical isotropic spectrum with three sharp peaks of almost equal intensity. Such a spectrum is shown by the broken line in fig. 15.
- b) M 12-NSE is practically insoluble in aqueous media. The spectrum of 2nmoles of M 12-NSE, if present as a film on the surface of the glass sample tube, shows no evidence of spin labels in solution.
- c) Fatty acid spin labels like M 12-NSE become incorporated into lipid bilayers (Raison *et al.*, 1971; Butler *et al.*, 1974) such that their long axes are oriented perpendicular to the plane of the bilayer (Seelig, 1970; Hubbell and McConnell, 1971).
- d) When incorporated into DMPC bilayers, M 12-NSE yields a typi-





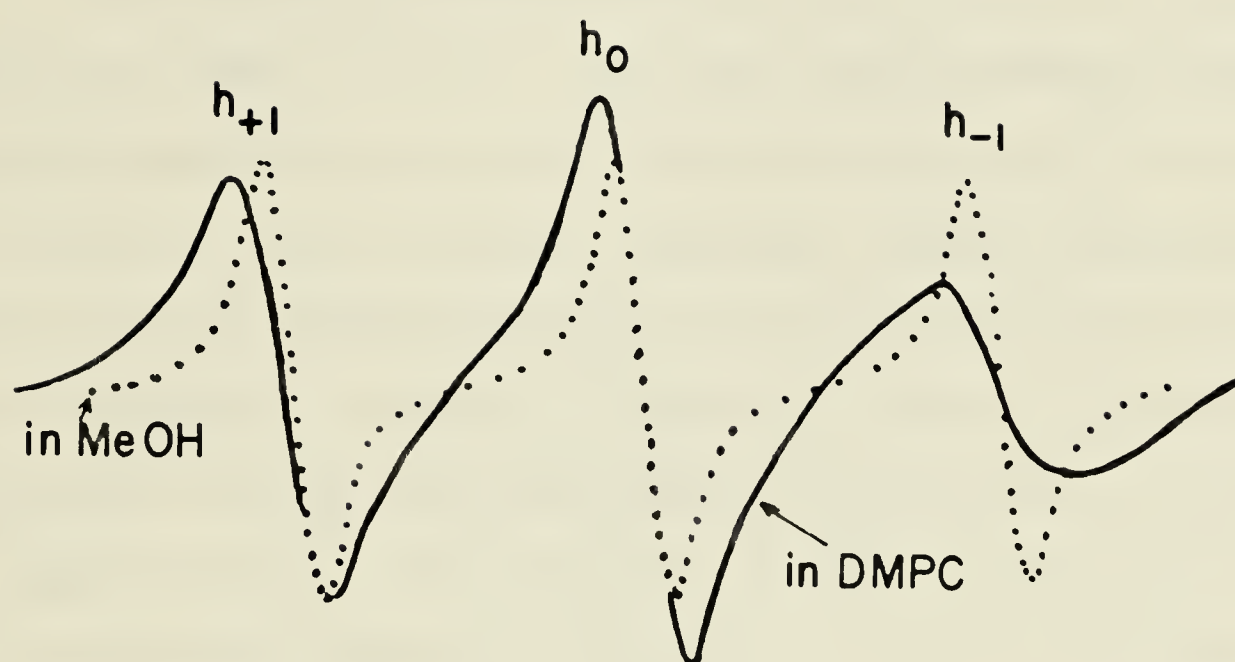


Fig. 15. Types of spectra. M 12-NSE was allowed to diffuse into DMPC liposomes over 10 min at 37°. Samples were introduced into sample tubes which were sealed. Spin labelled liposomes (—) yields a noticeably broader spectrum than does M 12-NSE in solutions in methanol.



cal spectrum that is quite distinct from that of a nitroxide label in solution. In Fig. 15 this is shown by the unbroken line superimposed on the isotropic spectrum of a label in methanol solution (the broken line).

The preliminary ESR experiments yielded a variety of spectra, and these are shown in Fig. 16. From some labelled membrane samples the spectrum was similar to that of an unrestricted nitroxide spin label in solution, while the spectra from other labelled membranes were similar to the restricted spectra of a nitroxide spin label intercalated into a lipid bilayer. However, the majority of spectra from membranes labelled with M 12-NSE were unlike either of these cases, but appeared to be somewhere between these extremes. A number of experimental protocols were therefore undertaken in attempts to resolve this problem, including a variation of the following labelling procedures; temperature at which the labelling was executed, the size of the membrane fragments that were labelled, the storage period of the labelled membrane prior to spectral investigation, the method of enzyme purification and the detergent treatment of the membranes. In spite of this, the type of spectrum that resulted could not be predicted. This problem led to development of the "controlled" labelling technique that was described in the chapter on methodology. With a fixed quantity of spin label present as a film in the sample tube, it was possible to study the effects of different probe:protein ratios with a small amount of M 12-NSE, which at the time these studies were undertaken could not be obtained commercially.

An examination of the results of more than fifty subsequent experiments, revealed that the assorted spectra that were obtained from



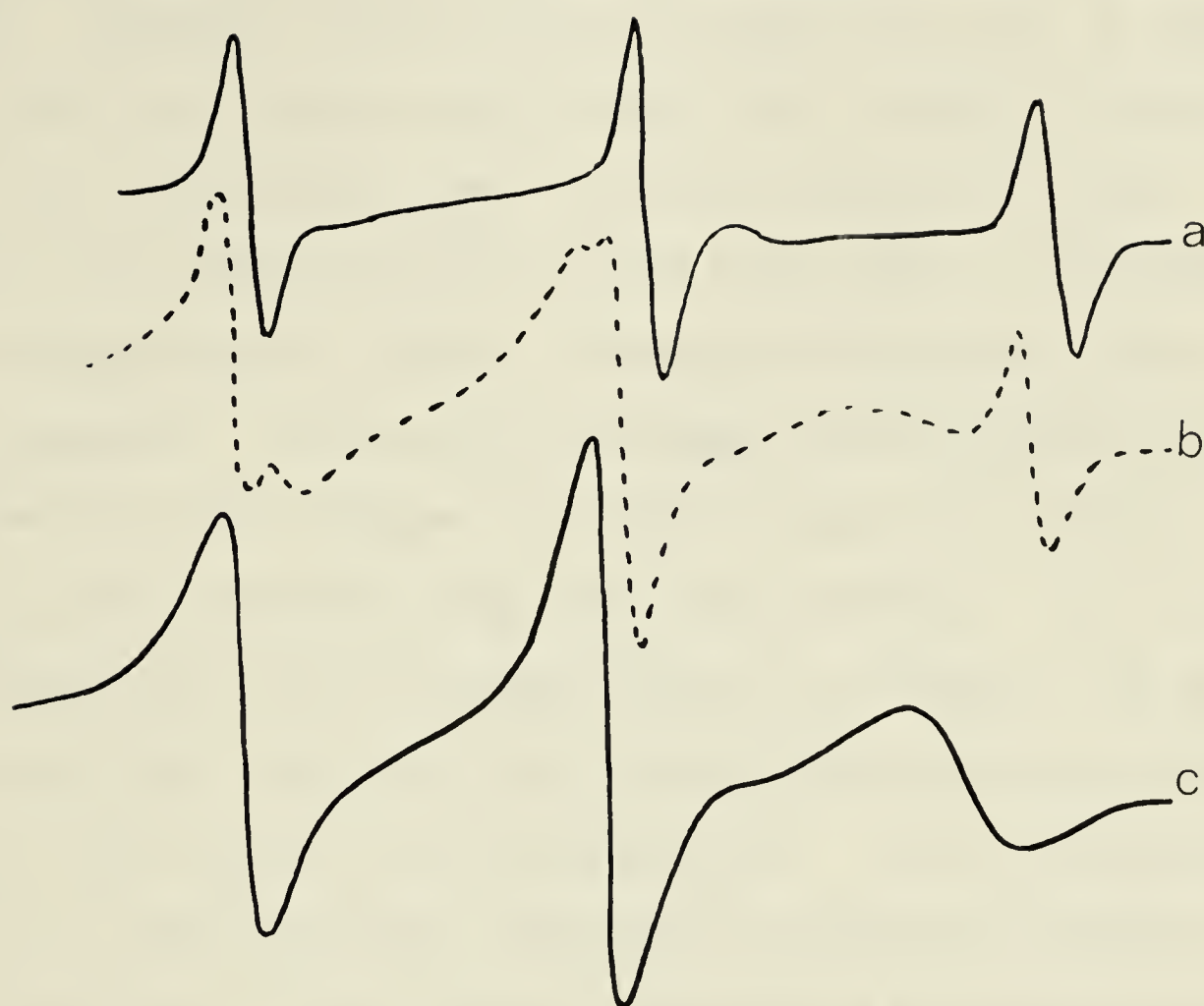


Fig. 16. Types of spectra from ox brain microsomes labelled with M 12-NSE. M 12-NSE was allowed to diffuse into ox brain membranes over 10 min at 37°. The spectra obtained are one of three types, being sharp or unrestricted (top trace, a), mixed (dotted middle trace, b) and broad or restricted (bottom trace, c).





spin labelled ( $\text{Na}^+ + \text{K}^+$ )-ATPase containing membranes could be grouped under three headings, which are designated unrestricted, restricted and mixed. Examples of these spectra are shown in Fig. 16a, b, and c.

The unrestricted spectrum shown in Fig. 16a is similar to but not identical with, that obtained from M 12-NSE in solution in methanol. Conversely, the restricted spectrum shown in Fig. 16c, is quite different from the unrestricted spectrum, although the three peak symmetry is still apparent. The peaks are now broader and very similar to those obtained with DMPC liposomes, presumably because the nitroxide containing probe experiences some restriction in motion. Fig. 16b shows a mixed spectrum, which as the name implies is a mixture of the two extreme positions, namely the unrestricted and the restricted spectra shown in fig. 16a and fig. 16c respectively. In the mixed spectrum there is a broad absorption, particularly between the low-field and the mid-field peaks. At this stage of the project, the appearance of this secondary peak was random, and its intensity apparently uncontrollable. Attempts to correlate the variation of this peak with any of the parameters that had been examined did not succeed. However, it was recognized that the mixed spectrum reflected both the unrestricted and the restricted spectra. The following section describes experiments carried out in an attempt to resolve this problem.

#### 4.2 Spectral variation and enzyme activity<sup>1</sup>.

Using a constant amount of M 12-NSE in each sample tube, a variety of membrane preparations of differing specific activity were

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<sup>1</sup> Some of the data presented in this section have appeared in press: Almeida and Charnock (1977) Biochim.Biophys.Acta 467, 19-28. (See appendix VII).



labelled. At least two membrane concentrations from each preparation were used. Fig. 17 shows a representative sample of some of the different types that were obtained. As was found previously, M 12-NSE will diffuse rapidly from the glass surface into the microsomal membrane. The shortest exposure time that was examined was 2 min at 37°C, after which time no further transfer occurred. In Fig. 17a, a 2-fold difference in membrane concentration (1.25 and 2.5  $\mu\text{g}$  protein/sample tube) is compared. The spectra from both samples have three sharp peaks equally spaced and of similar intensity, not unlike the isotropic spectrum of a rapidly tumbling nitroxide label in a solvent of low viscosity (Jost *et al.*, 1972). This type of spectrum, for which rotational correlation times can be readily determined (McConnell, 1956; McConnell and McFarland, 1970; Raison *et al.*, 1971), is characteristic of relatively unrestricted spin labels in the membrane. The lines in the two spectra have very similar intensities, but the low membrane concentration has some distortion of the base line due to residual M 12-NSE on the surface of the sample tube.

In Fig. 17b, a 4-fold difference in concentration of membrane protein, (4.0 and 16.0  $\mu\text{g}$  protein/sample tube) is compared. Here again the spectra are similar in appearance and resemble the isotropic spectrum described above. There is, however, a difference in the two spectra. A broad absorption is now apparent. This secondary peak is especially noticeable between the low-field and the mid-field peaks. Furthermore it is obtained with the higher membrane concentration, 16  $\mu\text{g}$  protein/sample. However, since the label concentration was the same for both samples, it follows that this phenomenon is only observed at the



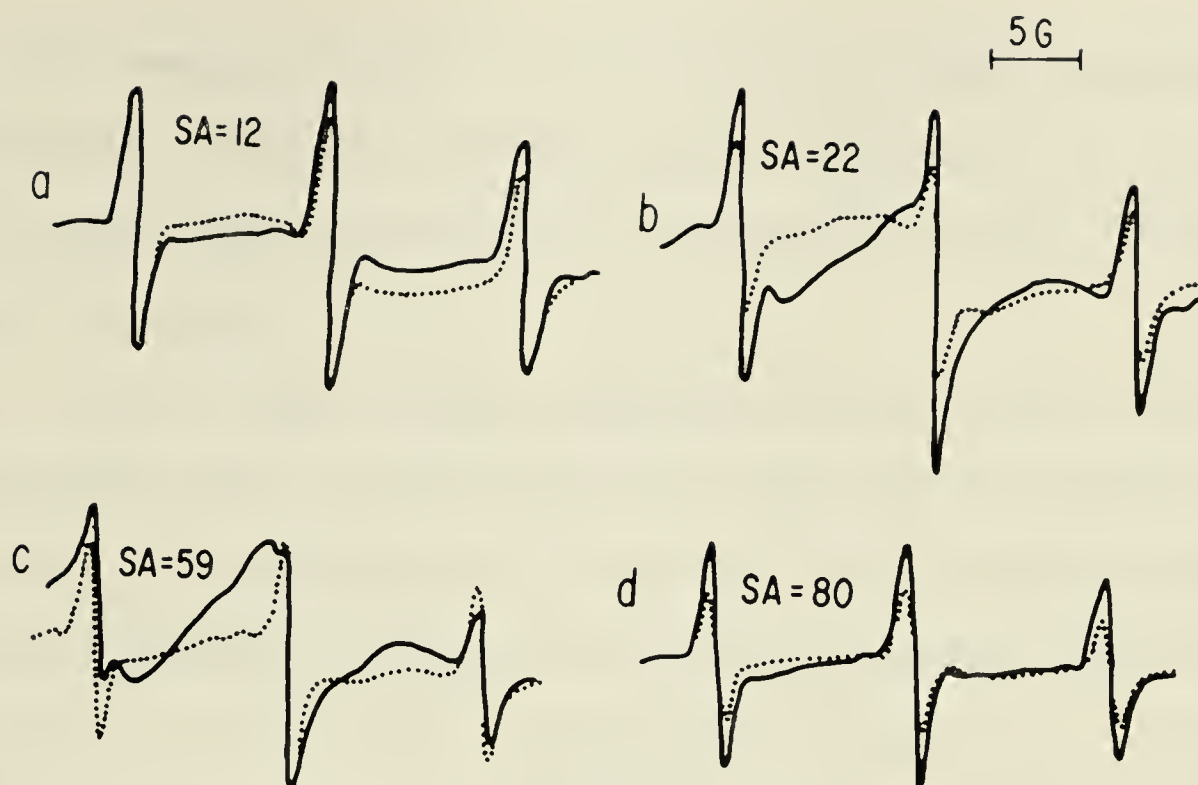


Fig. 17. M 12-NSE in ox brain microsomes. Untreated ox brain microsomes (a,b,c) and after 2 min extraction with 0.1% sodium deoxycholate in the presence of 2mM ATP and 80mM NaCl (d) were labelled in sample tubes containing 2 nmoles M 12-NSE. Diffusion of the probe was allowed to proceed for at least one hour prior to spectral examination. Two membrane concentrations from each of the 4 enzyme preparations were examined. The specific activities (S.A.) of the preparations were respectively 12, 22, 59 and 80  $\mu\text{moles Pi/mg protein/hr}$  as shown on the figure. The membrane concentrations in  $\mu\text{g protein/sample}$  were as follows, and in each set of data the lower concentration is given as the broken line: (a), 1.25 (.....) and 2.5 (—); (b), 4.0 (.....) and 16.0 (—); (c), 6.6 (.....) and 21.0 (—); (d), 2.6 (.....) and 25.5 (—).





lower probe:membrane ratio ( $1.2 \times 10^{-4}$   $\mu$ moles M 12-NSE:1 $\mu$ g protein). Therefore the increase in membrane concentration from 4.0 to 16.0  $\mu$ g protein/sample (the only variable) is responsible for the alteration in spectral line shape.

Fig. 17c shows another experiment in which a pair of spectra were obtained with a 3-fold difference in membrane concentration from 6.6 to 21.0  $\mu$ g protein/sample. At the lower membrane concentration, the spectrum is typically isotropic, but when the membrane concentration in the sample is raised to 21.0  $\mu$ g protein the spectrum is no longer isotropic. The divergence in spectral line shape is by far the greatest in this pair of spectra although the difference in membrane concentration is intermediate to that used in the two previous experiments, which was 2-fold and 4-fold respectively (fig. 17a and fig. 17b).

Finally, in Fig. 17d a pair of spectra are shown that were obtained by spin-labelling ox brain microsomes that had been extracted with DOC but in the presence of sodium and ATP. The line shape of both spectra are now typically isotropic despite the fact that the membrane concentration of the two samples differs by an order of magnitude from 2.6  $\mu$ g protein to 25.5  $\mu$ g protein per sample tube.

From this series of paired experiments it is clear that a number of factors influence the spectrum that will result when  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  membranes are spin labelled. First, the ratio of the number of spin labels and the amount of membrane expressed as the protein concentration, directly influences the spectrum. Second, if the first three pairs of spectra in fig. 17 are examined collectively, it is apparent that in addition to the membrane concentration, the specific activity of the enzyme preparations also influences the spectrum. Finally it



appears that detergent treatment, in this case DOC, can alter both of the above parameters. On the one hand a 10-fold increase in membrane concentration did not alter the shape of the spectrum; on the other hand, although the detergent treatment yielded a more pure membrane, this increased specific activity of the enzyme also did not alter the line shape of the spectrum. That is to say, despite an increased activity as well as an increase in the concentration of the membrane, there is no change in the quality of the spectrum.

#### 4.3 Spectral variation and membrane (protein) concentration<sup>1</sup>.

The effects of membrane concentration on spectral type were investigated by examining the spectra of samples containing a fixed amount of probe (2nmole) and varying amounts of membrane from a single enzyme preparation. A selection of these spectra is shown in Fig. 18. Although the spectra again vary with the membrane concentration, they can be grouped into the three categories of unrestricted, restricted and mixed which was described previously (see section 4.1). At the low membrane concentrations the spectrum is a typical isotropic spectrum characteristic of unrestricted labels. At the very high membrane concentrations, the spectrum is considerably broadened and now resembles the line shape of the spin-labelled DMPC liposomes (shown in fig. 15). At intermediate membrane concentrations, the shape of the spectrum is a superposition of the spectra at the extremes, with the relative intensities being determined by the membrane concentration. Thus at low membrane concentrations, the probe binds to sites that permit greater

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<sup>1</sup>Some of the data presented in this section have appeared in press: Almeida and Charnock (1977) *Biochim.Biophys.Acta.* 467, 19-28, (See appendix VII).



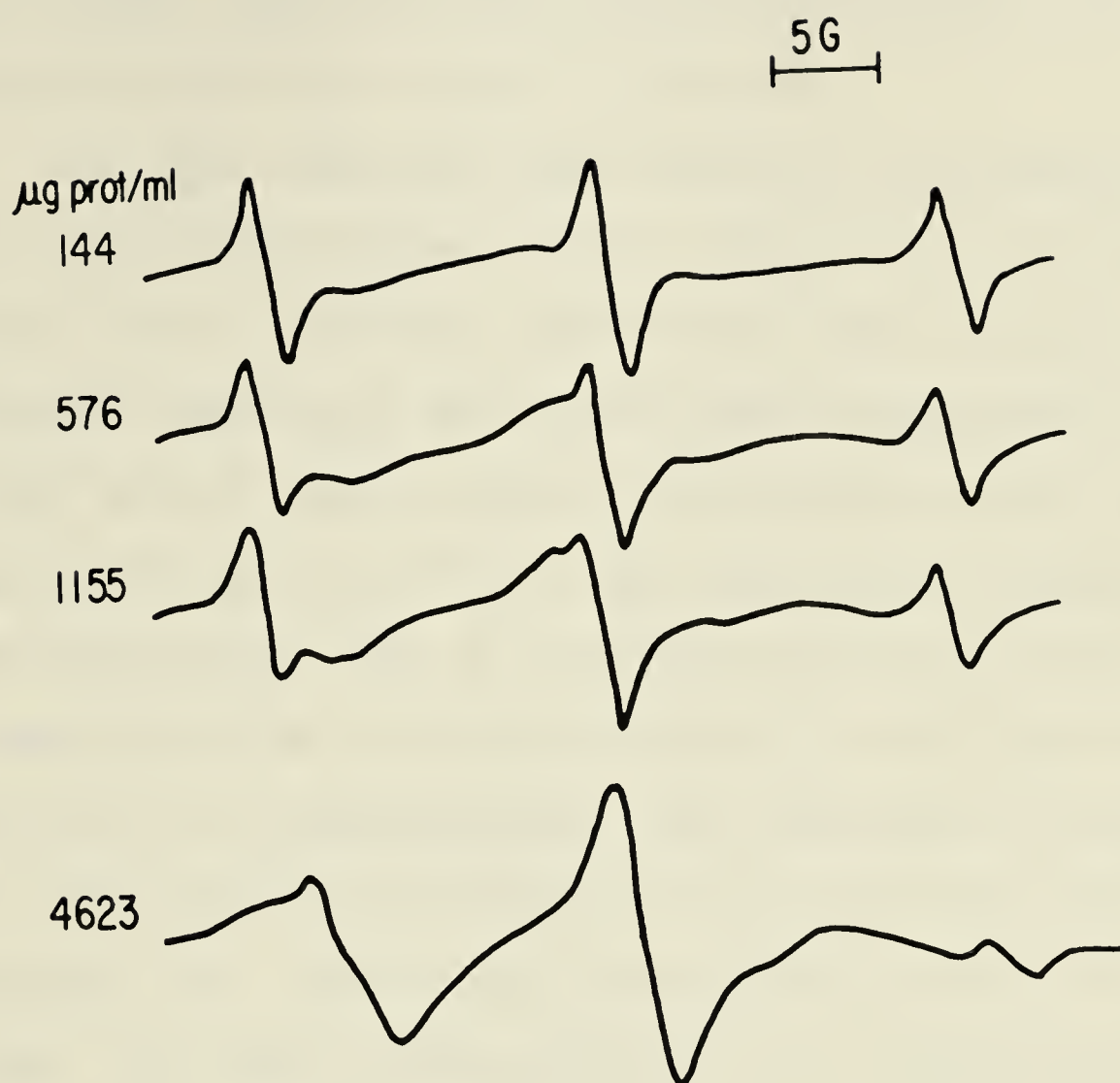


Fig. 18. The effects of membrane concentrations ( $\mu\text{g}$  protein) on the spectrum of microsomes labelled with M 12-NSE.

Increasing concentrations of untreated ox brain microsomes were added to sample tubes each containing 2 nmoles of M 12-NSE. Diffusion of the probe was allowed to proceed for at least one hour prior to spectral examination. Note the change in spectrum from sharp to broad as the concentration of the membrane increases.





motional freedom, whereas at high membrane concentrations a site that restricts the motion of the nitroxyl is preferred.

From these results and those presented in the preceding section (4.2) an important observation is made: The exact ratio of probe to membrane at which a restricted spectrum is obtained is constant for any one microsomal preparation, but may vary from one preparation to another. It is recalled that results presented in the previous section had shown that the specific activity of the enzyme preparation influenced the line shape of the spectrum. Thus it is possible that this variation probably bears some correlation to the specific activity of the enzyme preparation and also to the method employed in the purification procedure.

Spectra which are intermediate to the two limiting types described above, are a separate group constituting a mixture of two spectral types. They show the three peaks which are characteristic of the three sharp line spectrum and also display evidence of intermediate peaks. At present these mixed spectra cannot be characterized further. Presumably they arise if both restricted and unrestricted labels contribute to the spectrum.

#### 4.4 Effect of enzyme purification on the spectrum<sup>1</sup>.

The experiments that have been discussed in the preceding sections strongly suggest that a correlation might exist between enzyme purity and spectral type. Thus it seemed pertinent to examine the

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<sup>1</sup>Some of the data presented in this section have appeared in press: Almeida and Charnock (1977) *Biochim.Biophys.Acta* 467, 19-28 (See appendix VII).



spectra obtained when a highly purified (most active) membrane preparation was spin labelled. Of the methods that were used for the purification of this enzyme, (and these have been described in the chapter on methods) extraction with SDS was the most effective in increasing the specific activity of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  containing membranes. Although the mean values of such preparations was in the range of about 100  $\mu\text{moles Pi/mg protein/hr}$ , several preparations did attain a level of about 150  $\mu\text{moles Pi/mg protein/hr}$ . Three such high activity enzyme preparations were spin labelled and examined for their spectral characteristics. Fig. 19 shows the results obtained from a membrane preparation that had a specific activity of 158  $\mu\text{moles Pi/mg protein/hr}$ . Four different membrane concentrations were labelled with a constant amount of 2 nmoles of M 12-NSE. Although the amount of membrane added was varied from 3.4 to 23  $\mu\text{g protein}$ , it is quite clear that the spectra are all of the restricted type. If the peak height is plotted against membrane concentration, Fig. 20, a linear plot is obtained for the first three points. This relationship supports homogeneous labelling in this system. The highest membrane concentration shows a flattening of the curve, presumably because the amount of M 12-NSE is now the limiting factor. A total of three SDS-treated preparations with elevated specific activities of 158, 144 and 142  $\mu\text{moles Pi/mg protein/hr}$ , were spin labelled and examined in this manner. In all cases the restricted spectrum was observed with no evidence of mixed or unrestricted spectra.

It is apparent that high activity preparations appear to be homogeneously labelled in that only a single spectral type is obtained. Therefore it is possible that restricted spectra result from the binding of spin labels to distinct sites (R sites). By implication a second



Fig. 19. The effects of membrane concentration after enzyme purification. Increasing concentrations of sodium dodecyl sulfate extracted ox brain microsomes were added to sample tubes each containing 2 nmoles M 12-NSE. Diffusion of the probe was allowed to proceed for at least one hour prior to spectral examination. Spectral comparison of samples containing 22.8 (.....), 11.9 (.-.-.-), 6.9 (----) and 3.4 (——)  $\mu\text{g}$  protein per sample tube.





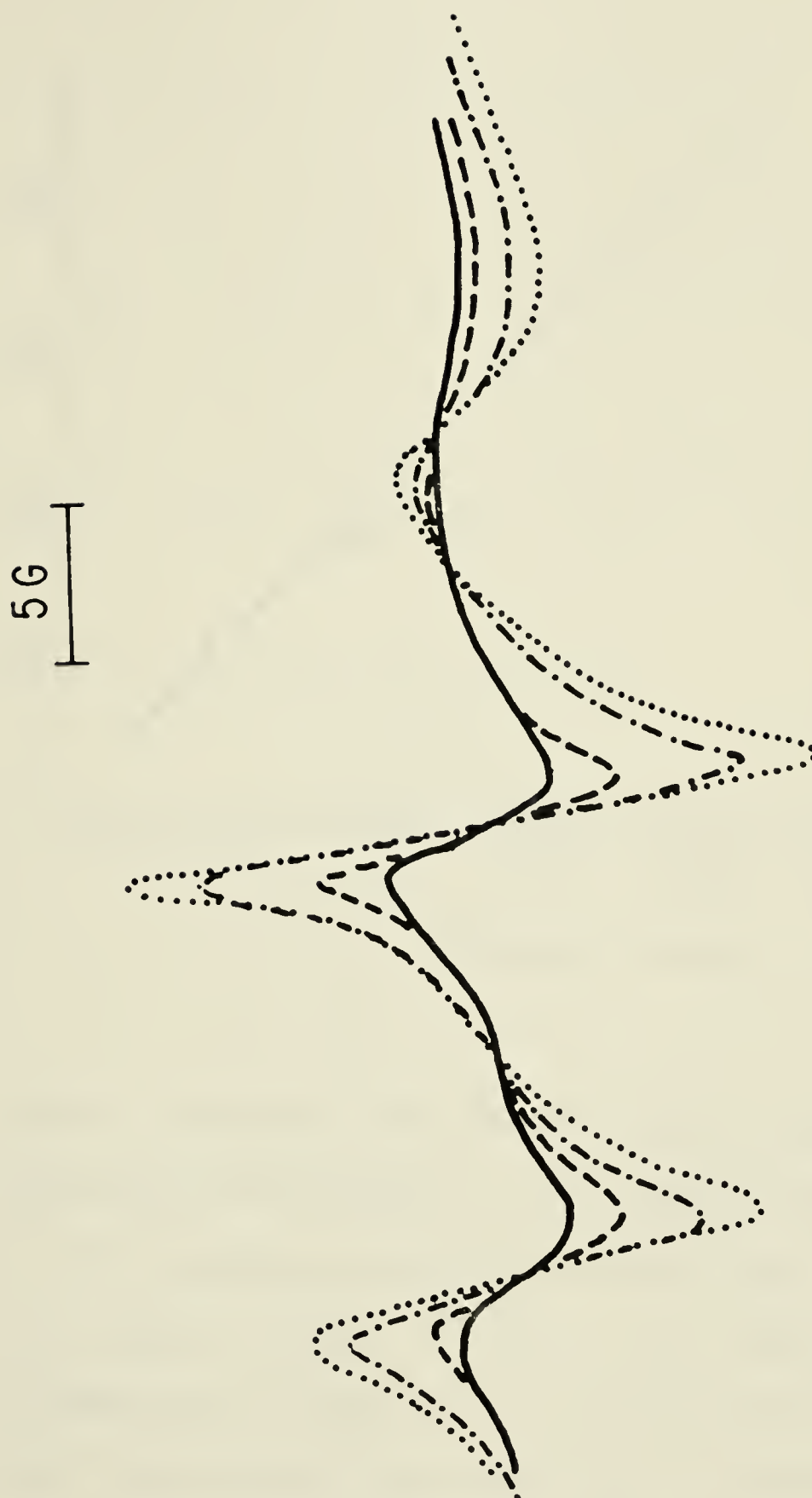


Fig. 19. Legend on separate page.



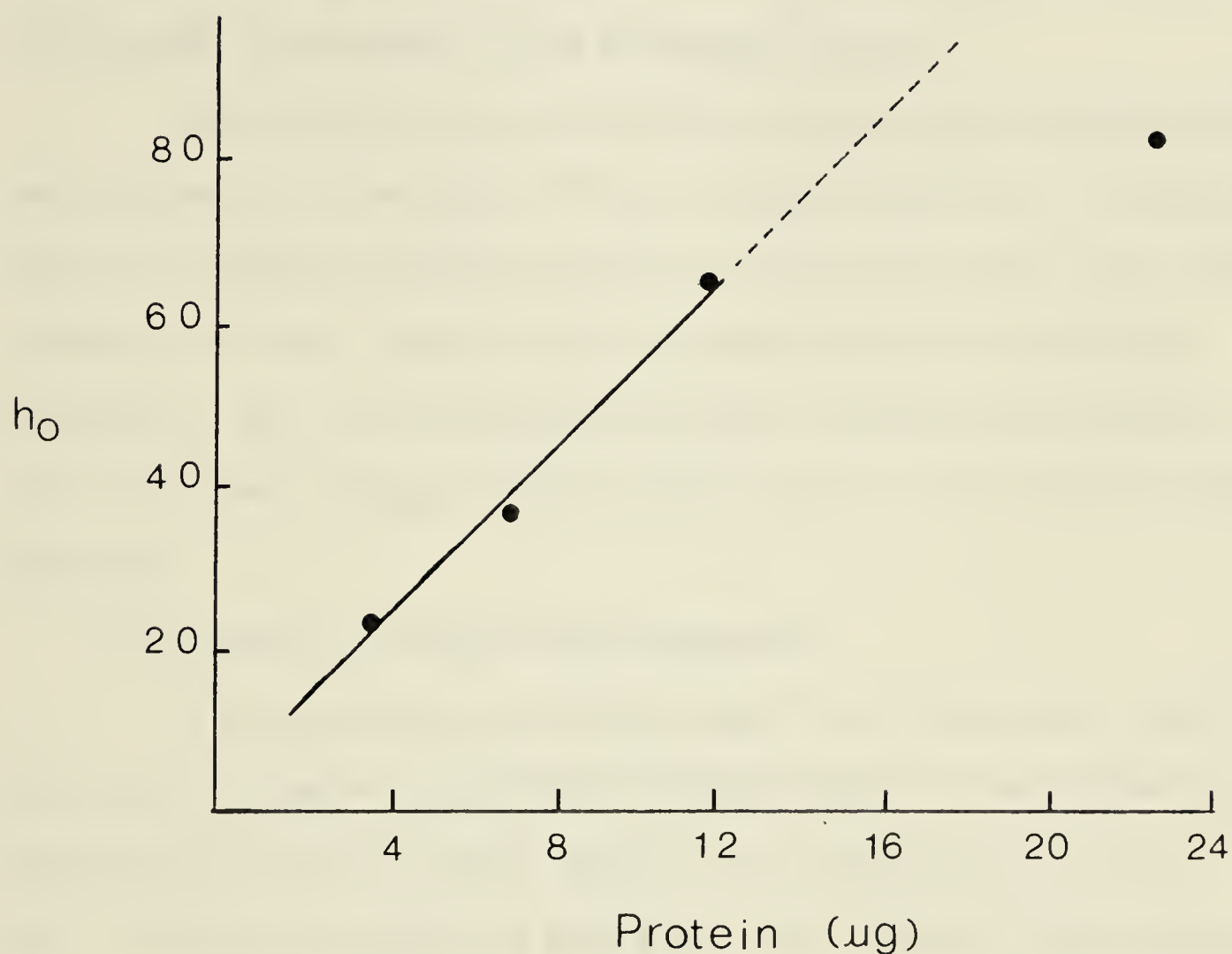


Fig. 20. Spectral homogeneity with membrane (protein) concentration. Increasing concentrations of sodium dodecyl sulfate extracted ox brain microsomes were added to sample tubes each containing 2 nmoles M 12-NSE. Diffusion of the probe was allowed to proceed for at least one hour prior to spectral examination. The mid-field peak height ( $h_0$ ) is plotted versus protein concentration. There is no change in the profile of the line if the alternate parameter ( $h_0 \times w_0$ ) is used in place of  $h_0$ .



population of M 12-NSE binding sites would be responsible for generating unrestricted spectra (U sites), while a mixture of R and U site labelling would result in a mixed spectrum. This concept is also supported by the results presented in the preceeding section.

Applying this two site concept to the study described above, two conclusions can be drawn: first, the purification of ox brain membranes by treatment with SDS results in a loss of U sites and a concentration of R sites; second, this treatment results in a parallel increase in specific activity and R sites, inviting the suggestion that the R sites are closely related to the function of the protein macromolecule.

#### 4.5 Spectral variation and temperature

The preceding experiments support the premise that the different types of spectra arise from M 12-NSE binding to two distinct sites. Furthermore, they also define some of the characteristics of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  containing membranes, which influence the binding of this probe. Since an important characteristic of this enzyme is its thermal sensitivity, an examination of the effects of temperature on the different spectral categories that have been defined was considered essential. Fig. 21 shows the effects of temperature on the three types of spectra. Microsomes labelled predominantly at the U sites (Fig. 21a) undergo a loss of peak intensity when the temperature is reduced. This line broadening at lower temperature is most pronounced on the high field line. The extent of this change is from 1.3 at  $37^\circ$  to 1.81 at  $5^\circ$  or 138% of the value obtained at the reference temperature, if measured as a ratio of mid- and high-field ( $h_0/h_{-1}$ ) peaks, or  $1.5 \times 10^{-10}\text{s}$  at  $37^\circ$  to  $3.6 \times 10^{-10}\text{s}$  at  $5^\circ$  or 240% when measured as tumbling times  $\tau_0$ .





Fig. 21 The effects of temperature on the different types of spectra. Ox brain microsomes having differing specific activities of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  ( $\mu\text{moles Pi/mg protein/hour}$  at  $37^\circ$ ) were labelled with M 12-NSE as described in the text, and their spectra examined at different temperatures. Preparation (a), specific activity  $13 \mu\text{moles Pi/mg protein/hr}$ ;  $1250 \text{ ng enzyme protein/nmole M 12-NSE}$  examined at  $38^\circ$  (—) and at  $5^\circ$  (.....). Preparation (b), specific activity  $82 \mu\text{moles Pi/mg protein/hr}$ ;  $1515 \text{ ng enzyme protein/nmole M 12-NSE}$  at  $38^\circ$  (—) and at  $5^\circ$  (.....). Preparation (c), specific activity  $49 \mu\text{moles Pi/mg protein/hr}$ ;  $3250 \text{ ng enzyme protein/mole M 12-NSE}$  at  $37^\circ$  (—) and at  $7^\circ$  (.....).



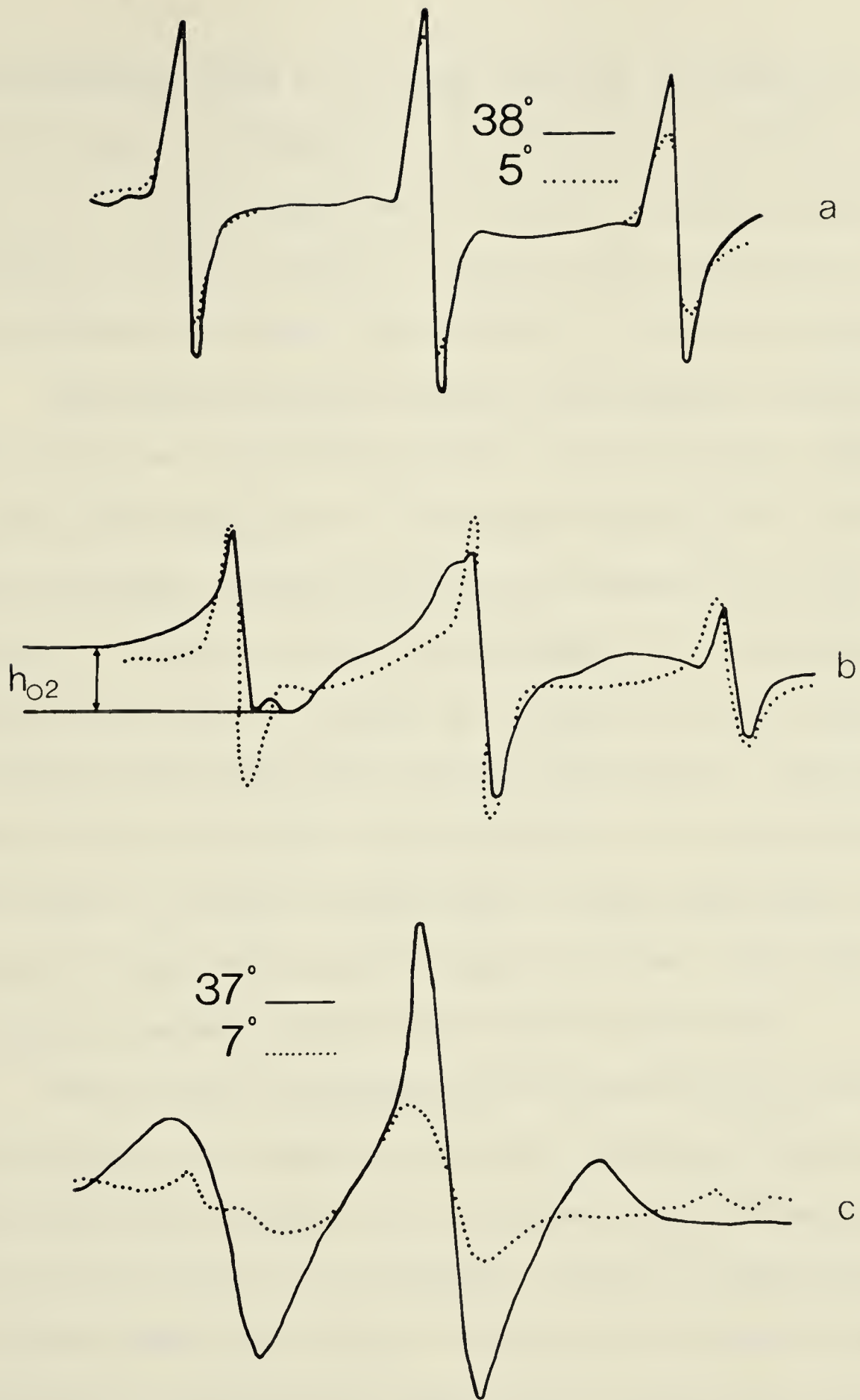


Fig. 21. Legend on separate page.



The corresponding values for M 12-NSE dissolved in methanol are 1.10 to 1.19 for  $h_0/h_{-1}$  or 108% and  $0.6 \times 10^{-10}$  s to  $1.0 \times 10^{-10}$  s or 166% for  $T_0$ , at 37° and 5° respectively. Thus for a similar change in temperature, M 12-NSE registers a greater change when bound to the U sites on ox-brain microsomes than when free in solution in methanol.

When the R sites are labelled, the changes in the spectrum due to cooling are considerably greater. As was observed with the U sites, Fig. 21C shows that at 7° the high-field peak ( $h_{-1}$ ) undergoes excessive reduction thereby diminishing the possibility of accurate measurement. Although at the low temperature the spectrum shows some evidence of labelling of U sites, it is important to note that these are relatively few judging from the peak intensities. From these observations it can be concluded that irrespective of the site in the membrane to which it binds, the spin label M 12-NSE experiences some loss of motional freedom when the microsomes are cooled. This effect is considerably more marked at the R sites than at the U sites.

The cooling of microsomes with mixed labelling results in spectral changes that cannot be measured as easily as those described above. On the one hand there is an apparent loss of intensity from labels at the R sites and a concomitant increase in contribution to the spectrum from labels at U sites. Secondly, as was pointed out above, the magnitude of the effect of temperature on the spectra obtained from U and R sites is not equal. Thus changes to the mixed spectrum caused by altering the temperature, cannot be readily applied to an understanding of fluidity changes in the membrane. However it is noted that the secondary peak,  $h_{02}$  which reflects probe binding to R sites, de-





creases with temperature. That cooling causes a decrease in the affinity of M 12-NSE binding to R sites is interesting, but in isolation is of little consequence to the present study. However it will be discussed again in a later section.

#### 4.6 Binding affinity of R and U sites.

In the study of the effects of membrane concentration on spectral type, the inference was made that the spin probe M 12-NSE displayed a preference for R sites (section 4.3 and Fig. 18). In this section, experiments which were designed to further examine this possibility, are described. Using a constant amount of M 12-NSE, (2 nmoles/25  $\mu$ l sample), three different amounts of membrane protein were labelled for each of three preparations with specific activities of 20, 35 and 56  $\mu$ moles Pi/mg protein/hr. All spectra were recorded at 37° and were found to be of the mixed type. The results which are presented as a histogram in Fig. 22, show how the height of the secondary peak,  $h_{02}$  (as shown previously by the double-headed arrow in Fig. 21b, varies with the specific activity of the preparation. Two observations can be made from the data given in Fig. 22.. First, as the membrane protein concentration is raised there is a corresponding increase in the intensity of the secondary peak  $h_{02}$ . That is to say, the number of R sites that are available for labelling increases with membrane concentration. Secondly, the labelling of R sites increases disproportionately as the enzyme specific activity of the microsomes is raised. This latter aspect is best seen by comparing the data from ox brain microsomes of lowest and highest activity, at the two extreme protein concentrations shown in Fig. 22. For a similar increase in membrane protein the two preparations register respectively about a 3-fold and a 6-fold



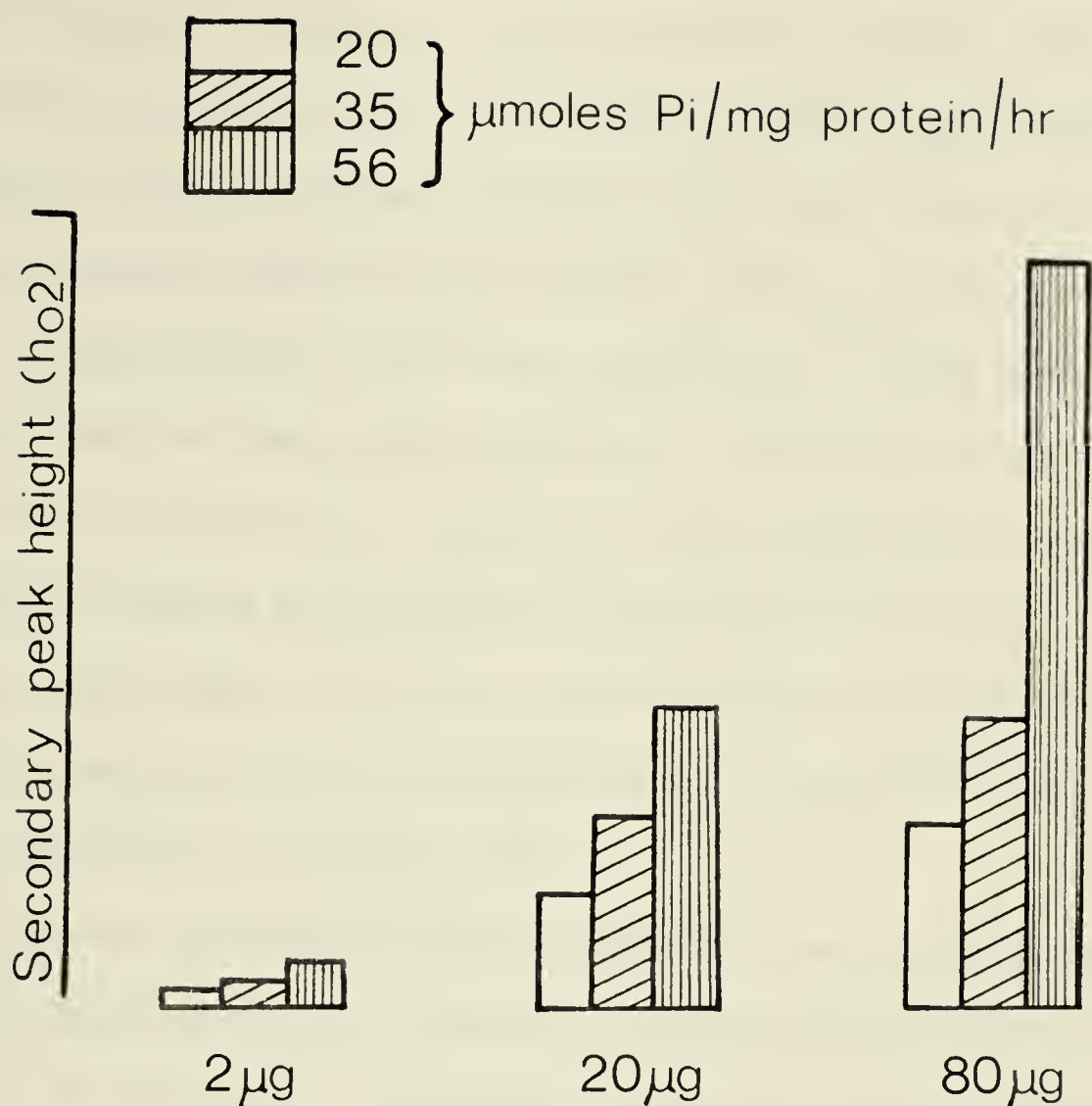


Fig. 22 Probe binding to microsomal R sites. Ox brain microsomes having different levels of specific activity of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase (shown on the figure) were labelled with 2 nmoles M 12-NSE to produce membrane concentrations of 2, 20 and 80 µg membrane/sample. Both the spin labelling of the membranes and recording of the spectra were conducted at 37°C. The height of the secondary peak h<sub>02</sub> (as shown in spectra Fig. 21b) was determined for each spectrum and is shown in mm on the vertical axis of the histogram.



increase in the numerical value of  $h_{O_2}$ . Since the concentration of M-12-NSE is fixed at 2 nmoles, it is reasonable to presume that competition for this label occurs at the higher membrane concentration. Quite clearly, the simultaneous labelling of U and R sites in these experiments prevents precise determination of the affinities for the two sites. Nevertheless, under these conditions it would appear that of the two sites that have been identified, the binding of spin probe to the R sites is preferred. Support for this interpretation can be drawn from the results of experiments previously illustrated in Fig. 21b; these results show that there is an increase in binding to U sites only after a decrease in R site binding occurs due to cooling.

#### 4.7 The effect of ascorbic acid.

The data presented so far support the two-site hypothesis, but do not contribute very much information towards locating these sites. In the case of the R site a reasonable argument can be developed to suggest a location within the bilayer and possibly near the  $(Na^+ + K^+)$ -ATPase macromolecule. In contrast, information about the location of the U sites is much less definite. It is unlikely that unrestricted spectra arise from labels in solution as this label was shown to be practically insoluble in the aqueous buffer being used. Furthermore, when a spin-labelled microsomal suspension was resuspended in sucrose buffer and then re-sedimented by high speed centrifugation, not only was there no change in the spectral characteristics of the particulate enzyme but the clear supernatant was devoid of any spectroscopic signal. Thus it would appear that the spectra which have been described arise only from spin labels associated with the membrane in some manner. In





this section experiments designed to further characterize this association are described.

Spin labels are rapidly reduced by ascorbic acid and this property has been used to differentiate between spectral peaks produced by labels in solution, from those peaks derived from membrane bound labels (Kornberg and McConnell, 1971; Grant and McConnell, 1973). The results of this investigation are shown in Fig. 23, where it is apparent that M 12-NSE in methanol solution is rapidly reduced by ascorbic acid and this is generally true for nitroxides in solution (Kornberg and McConnell, 1971; Grant and McConnell, 1973). As determined by a measure of the peak height, more than half of the M 12-NSE in methanol solution is reduced within the first 10 min of exposure to 2 mM ascorbic acid. However, rates as rapid as 2 min for 90% reduction of signal have been reported in the literature but these rates are not directly comparable with the result shown here because they can be influenced by a variety of factors including the relative concentration of the nitroxide label to ascorbic acid, and the efficiency of mixing which is attainable in the sample chamber that is being employed.

In the case of the labels at the U sites the reduction in peak height is only half as fast as that seen in the methanol solution, requiring about 20 min for a 50% loss of signal. This difference in sensitivity to ascorbic acid implies that the labels at these U sites may not be freely accessible to this reducing agent. In marked contrast, the labels at the R sites are not significantly reduced by ascorbic acid within the first hour of exposure and only very slightly if the exposure period is monitored for a second hour thereafter. Further-



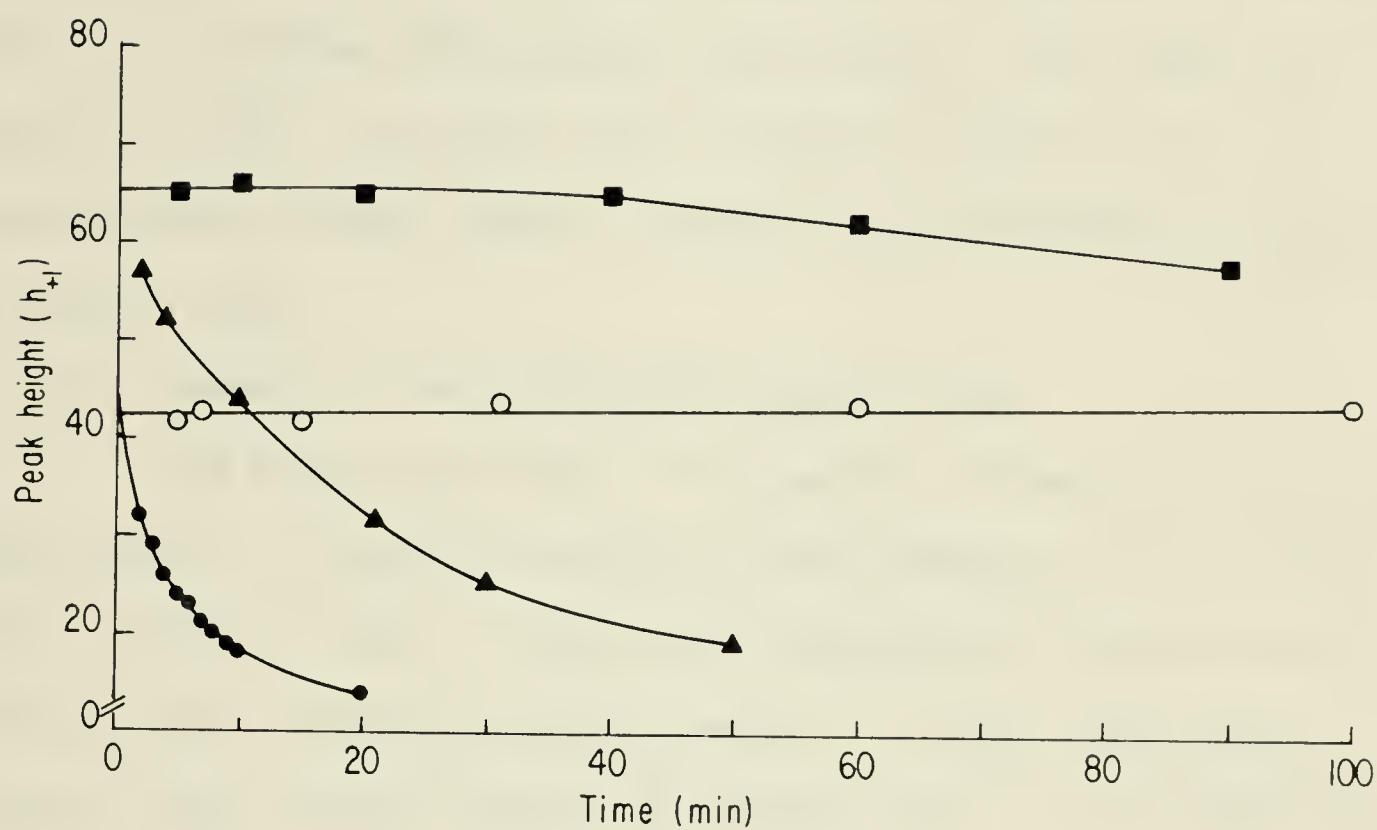


Fig. 23. The effect of ascorbic acid on the peak height of M 12-NSE in different environments. 2mM ascorbic acid buffered to ph 7.5 with Tris was added to M 12-NSE: in methanol (●); at U sites in ox brain microsomes (▲); at R sites in ox brain microsomes (■); and in DMPC liposomes 1 mg lipid/ml(○).



more, when the reduction process was conducted at 0° no potentiation of this slow rate was observed. This protection from ascorbic has been reported by others, and is cited as evidence for a labelled site deep within the membrane core (Kornberg and McConnell, 1971; Grant and McConnell, 1973). A similar very slow rate of reduction was also observed when M 12-NSE labelled liposomes made from DMPC were exposed to ascorbic acid.

#### 4.8 Summary of the evidence for U and R sites.

The results described in the section 4 show that M 12-NSE will bind to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enriched ox brain membranes at two distinct sites. If one or other of the sites is predominantly labelled then unrestricted or restricted spectra result. If both sites become labelled, then a mixed spectrum is obtained which is a superposition of the two types seen previously. A summary of the information contained in section 4 can be conveniently presented as a list of the characteristics of the two binding sites and this follows:

- a) Labelled U sites are characterized by a sharp spectrum similar to but not identical with rapidly rotating nitroxides in solution, and R sites are characterized by a broad spectrum similar to nitroxides within a lipid bilayer.
- b) Both sites are influenced by the ratio of the probe to membrane concentration; a high ratio results in the labelling of U sites while a low ratio results in R site labelling.
- c) Enzyme preparations with high specific activity yield spectra resulting from R site labelling while those with low specific activity yield spectra typical of U site labelling. It





is possible that very pure ( $\text{Na}^+ + \text{K}^+$ )-ATPase preparations lack U sites altogether but such preparations have not yet been obtained in this laboratory. However, mild treatment of ox brain membranes with DOC may cause an increase in enzyme specific activity without a loss of U sites.

- d) Both U and R sites report a loss of mobility when labelled ox brain membranes are cooled. The effect of temperature is considerably greater on the R sites.
- e) M 12-NSE has a higher binding affinity for the R sites than for U sites. That is, when a limited amount of spin label is available, R sites are preferentially labelled. In support of this observation, mixed spectra when cooled show a decrease in R site labelling and an increase in the labelling of U sites.
- f) Ascorbic acid, reduces labels at U sites fairly rapidly, but not as fast as labels in solution in methanol. M 12-NSE at R sites is relatively resistant to reduction by 2mM ascorbic acid.

Although the precise location of the U and R sites with respect to membrane topography is not known, their marked diversity as documented above, warrants a detailed study of the effects of temperature. These studies are presented in the following section.

## 5. The effects of temperature on spin labelled membranes

In the chapter on methods, section 4.7 contained a discussion of some of the quantitative approaches that have been used in biological applications of ESR. Some of the important points are pertinent to



this section and are reproduced below:

- a) A number of factors can contribute to the type of spectrum, and these in turn may influence the choice of method used for quantification of spectral changes.
- b) Six methods of quantification were compared using DMPC liposomes labelled with M 12-NSE, as a control lipid bilayer.
- c) From the investigation it was decided that when the spectrum is broad, like that of a probe in a synthetic bilayer, a ratio of the mid- and low-field peaks  $[h_0/h_{+1}]$  is an appropriate quantitative measure.
- d) When the spectrum is typical of a very mobile spin probe, comparable to a probe solution in methanol, the ratio of the mid- and high-field peaks  $[h_0/h_{-1}]$  is an appropriate quantitative procedure.
- e) The purpose of reporting a calculated value for an inflection point ( $T_c$ ) is only to emphasize changes in the slope of plots showing the effects of temperature on the spectrum.

### 5.1 The effects of temperature on U sites.

When U sites on ox brain microsomes are labelled with M 12-NSE the spectrum obtained is similar to that seen with these nitroxyl spin labels in solution, and the suggestion was made that one possible location might be the surface of the bilayer (Grant and McConnell, 1973). The uniqueness of this site may result from specific phospholipid head groups in this region which in turn could influence the transition temperature (Almeida and Charnock, 1977). Thus it was of peripheral interest to examine the effects of temperature on labelled U sites. Fig.



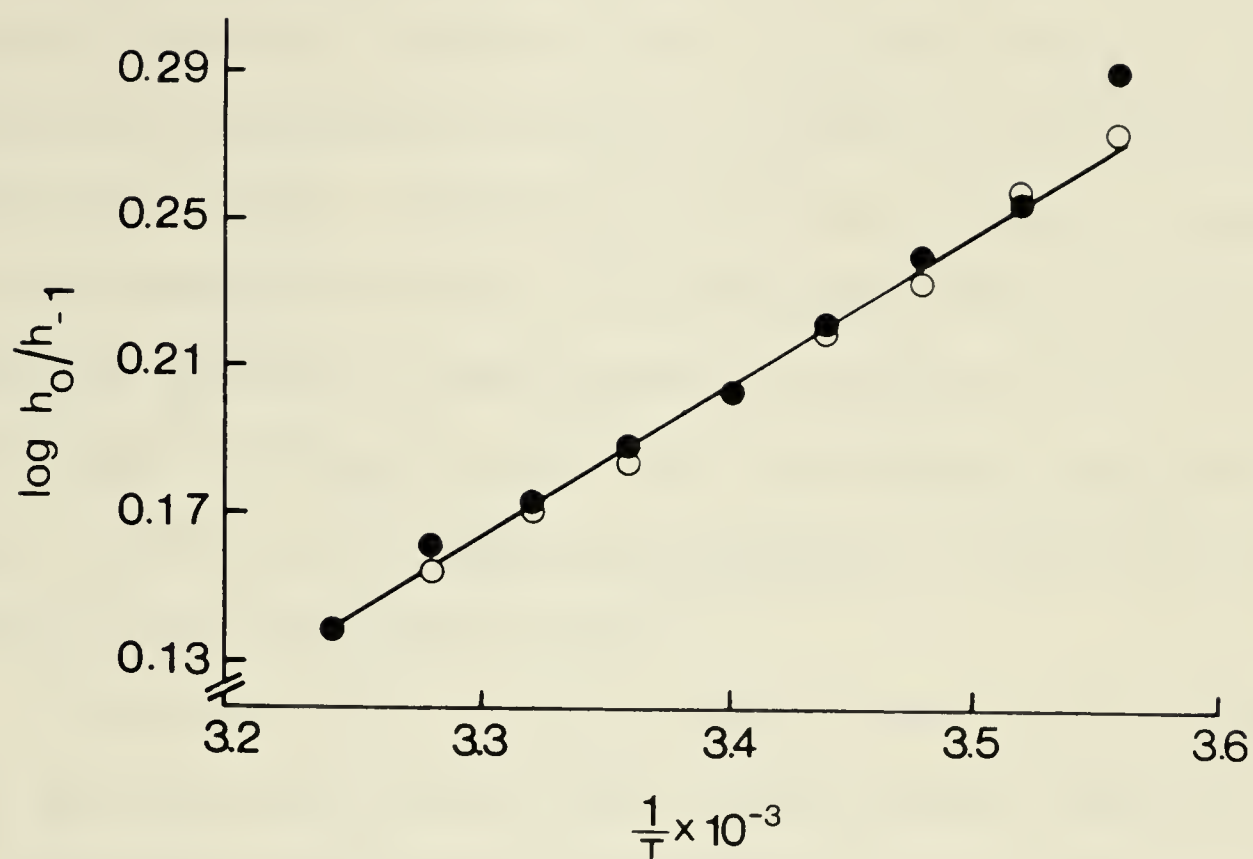


Fig. 24. Arrhenius plot of spectral changes from spin labelled unrestricted (U) sites on ox brain membranes labelled with M 12-NSE. The spin probe was allowed to diffuse into the membranes which were then equilibrated overnight at 4°C. About 30  $\mu$ l of the labelled membranes were introduced into capillary tubes (1.1 mm internal diameter) which were then sealed. Temperature alteration was achieved by cooling the sample in multiple steps of a few degrees between 37° and 5°C. The plots represent mean values of 9 determinations for each condition; (●) untreated membranes, and (○) sodium deoxycholate extracted membranes. A linear thermal response was found under both experimental conditions.





24 shows Arrhenius plots of the peak height ratio  $h_0/h_{-1}$  obtained from labelled ox brain microsomes, both before and after extraction with DOC. It is quite clear that M 12-NSE at U sites, although it reports a change in fluidity with temperature, provides no evidence of any discontinuity over the range examined. Furthermore, this linear response to temperature is not changed by extraction of the membranes with DOC although this treatment is known to remove membrane lipids and to result in a marked increase in the specific activity of the enzyme. However, it should be recalled that the temperature dependence of enzyme activity is not altered by this detergent treatment.

Thus while U sites definitely report a sensitivity to temperature, it does not appear that the label in this position reports any thermal transitions which could be attributed to changes in the physical properties of membrane lipids.

## 5.2 Limited study of the effects of temperature on R sites.

The results presented in section 4.2, 4.4, and 4.6 had shown a positive correlation between enzyme specific activity and the amount of R sites. Thus it is possible that selective retention of some specific lipid or lipid array might result from the purification procedures that were employed. Thus it would follow that spin labels at R sites, might report lipid sensitivities to temperature from a site in the membrane that is near or related to the enzyme protein macromolecule. On the strength of this supposition, the effects of temperature were examined on labelled R sites, using ox brain membranes both before and after extraction with detergents.

As shown in Fig. 25, when R sites are labelled, the spectrum is of the so-called 'broad' type similar to that obtained with lipo-



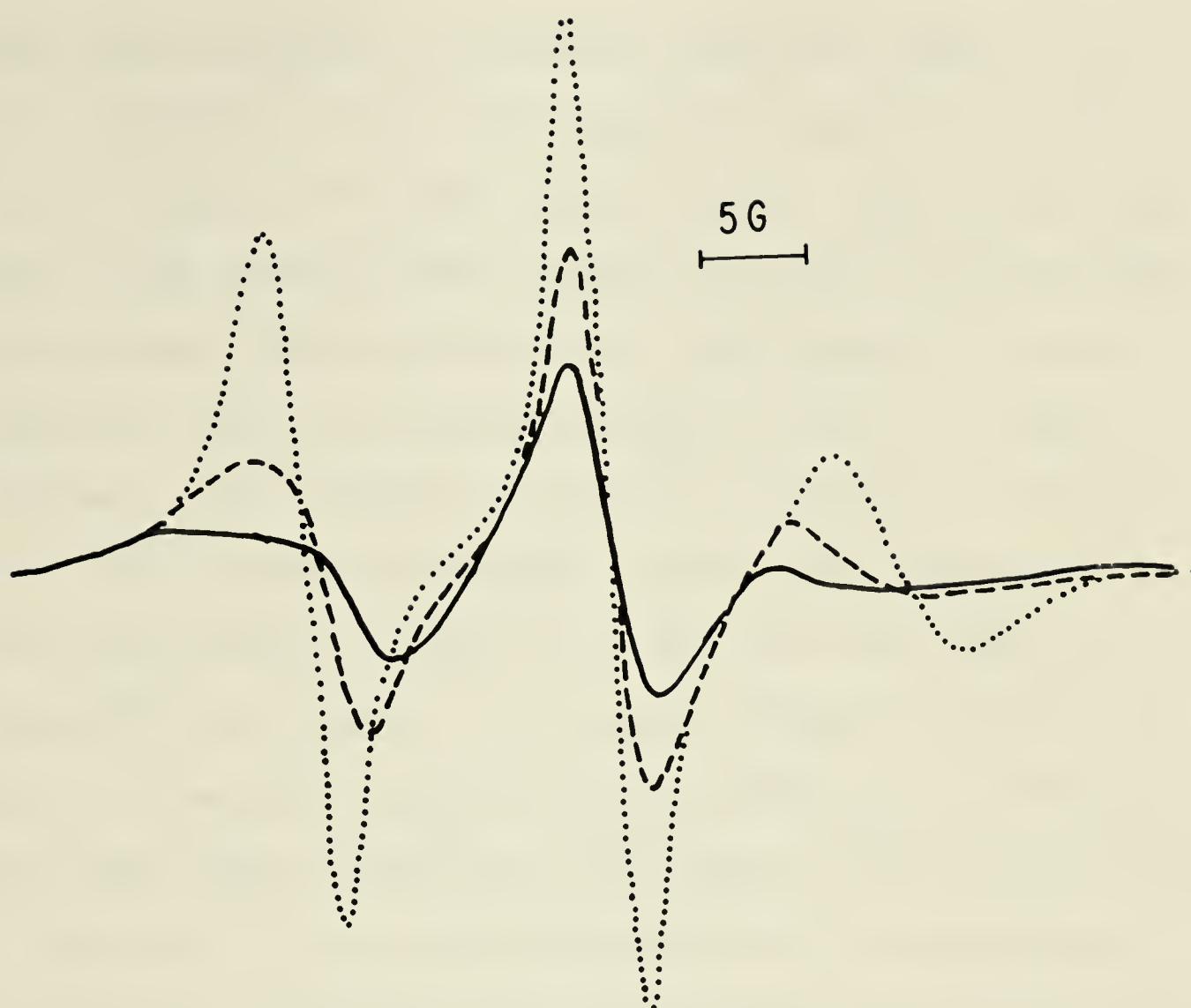


Fig. 25. The effects of temperature on the spectrum of M 12-NSE bound to restricted sites on ox brain membranes. M 12-NSE was allowed to diffuse into ox brain membranes which were then equilibrated overnight at 4°C. About 30  $\mu$ l of the labelled membranes were introduced into capillary tubes (1.1 mm internal diameter) which were then sealed. Temperature alteration was achieved by cooling the sample in multiple steps of a few degrees between 37°C and 5°C. Three representative traces obtained at 37° (· · · ·), at 19° (- - -), and at 7°C (—) are shown.



somes made from the synthetic phospholipid DMPC as was shown in Fig. 4 (p. 57). Furthermore, lowering the temperature results in a similar decrease in intensity of the three spectral peaks. Again, as was shown previously in the study with DMPC liposomes, the high-field peak experiences the greatest effect of temperature. Qualitatively it appears that labelled R sites in ox brain microsomes are similar to labelled DMPC liposomes. Fig. 26 gives the Arrhenius plots that were obtained with data from untreated and detergent treated enzyme preparations. Unlike the plots obtained for labelled U sites, it is very clear that none of the present plots is linear. The computer program described in section 2.8, p. 41 was used to obtain inflection points, all of which occur between 21-22°C, with no distinction with respect to the different treatments. The values for these inflection points and for the respective slopes of the plots above and below this point are very similar for the different treatments. This finding exactly parallels the effects of these detergents upon the thermal dependence of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity, but does not provide unequivocal evidence in support of the concept of increasing lipid specificity with increasing purification of the enzyme.

### 5.3 Extended study of the effects of temperature on R sites in untreated membranes.

The studies described above, rely upon a limited number of data points within the temperature range examined. Thus it is possible that secondary and perhaps less marked inflections might be obscured. These studies of the effects of temperature on M 12-NSE labelled ox brain microsomes have therefore been repeated in much greater detail.





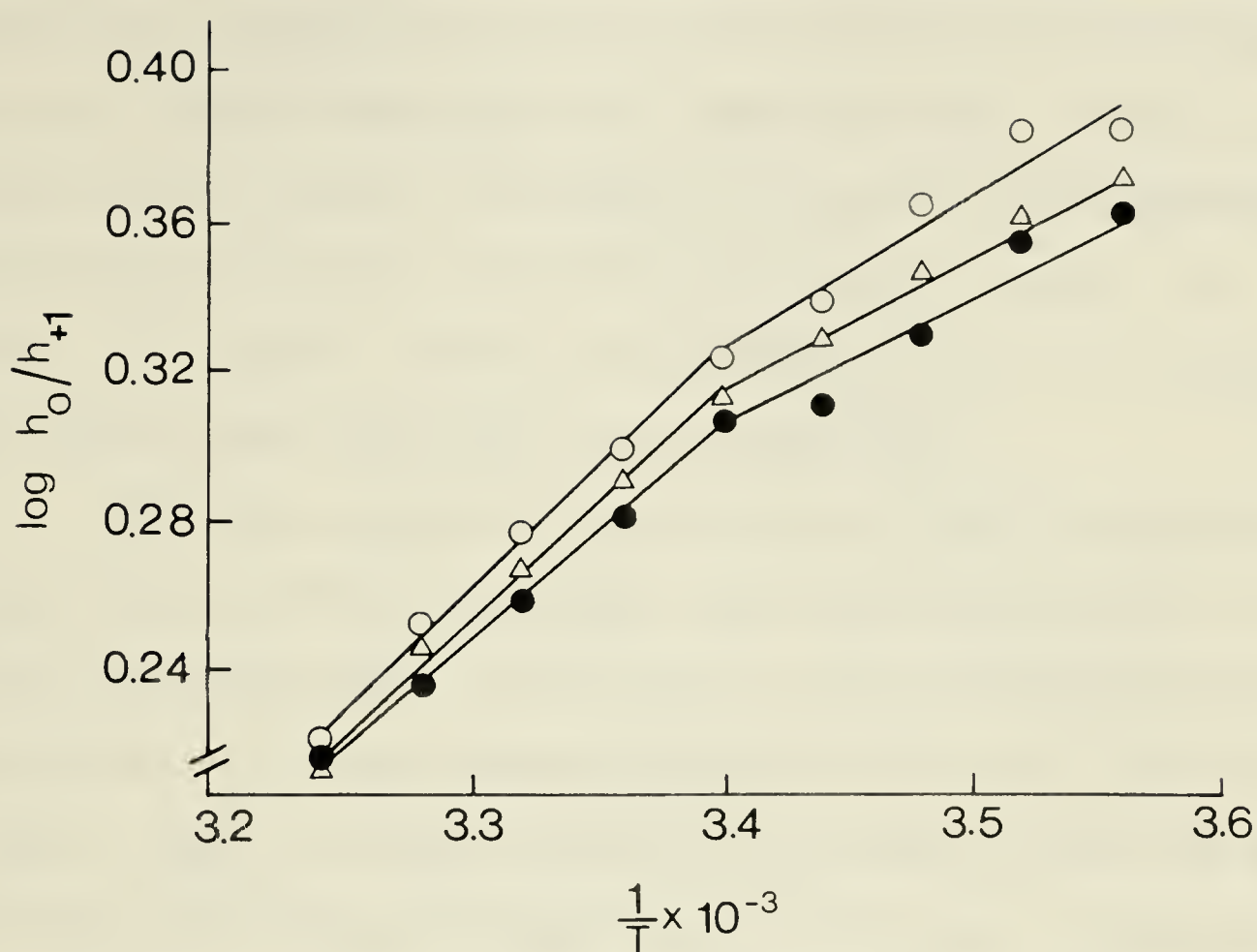


Fig. 26. A limited study of the effects of temperature and detergent extraction on restricted (R) sites labelled with M 12-NSE. The spin probe was allowed to diffuse into untreated ox brain membranes ( $\Delta$ ) and after extraction with sodium deoxycholate (O), or with sodium dodecyl sulfate ( $\bullet$ ), which were then equilibrated overnight at 4°C. About 30  $\mu$ l of the labelled membranes were introduced into capillary tubes (1.1 mm internal diameter) which were then sealed. Temperature alteration was achieved by cooling the sample in multiple steps of a few degrees between 37° and 5°C. The plots represent mean values of 4 determinations for each condition. A single discontinuity near 20° can be seen under all experimental conditions.



Arrhenius plots of data from untreated microsomes again revealed the characteristic non-linearity that was observed with the data obtained in the more limited studies described immediately above. However as was discussed earlier (section 2.8, chapter II, page 41), the presentation of data solely as Arrhenius plots might also conceal secondary inflections. Consequently the data are presented in Fig. 27 as a simple linear plot of peak height ratios versus temperature.

The results suggest at least two characteristic temperatures at about 10°C and 20°C with a possible third transition near 30°C. In the latter case, the effect is seen more as a step between 28°C and 30°C, without any real change in the slope of the line between 22°C and 37°C. That is, there is no major alteration in the rate at which the mobility of the probe changes with temperature in this range. On the other hand, at 20°C there is a marked change in the rate of probe mobility which is seen as a change in the slope of the line between 10°C and 20°C, compared to that above this point. This reflects a temperature range in which the mobility of the probe is markedly reduced and provides the basis for the two-state concept described in the earlier studies which employed a less extensive number of data points. It is noted further, that at about 10°C there is an even more noticeable inflection point indicating a very marked loss of probe mobility below this temperature.

#### 5.4 Extended study of the effects of temperature on R sites in DOC and SDS treated membranes.

Detergent treated preparations were also examined in similar detail. Again, simple linear plots of the peak height ratios versus temperature were drawn and are shown in Fig. 28. The data obtained



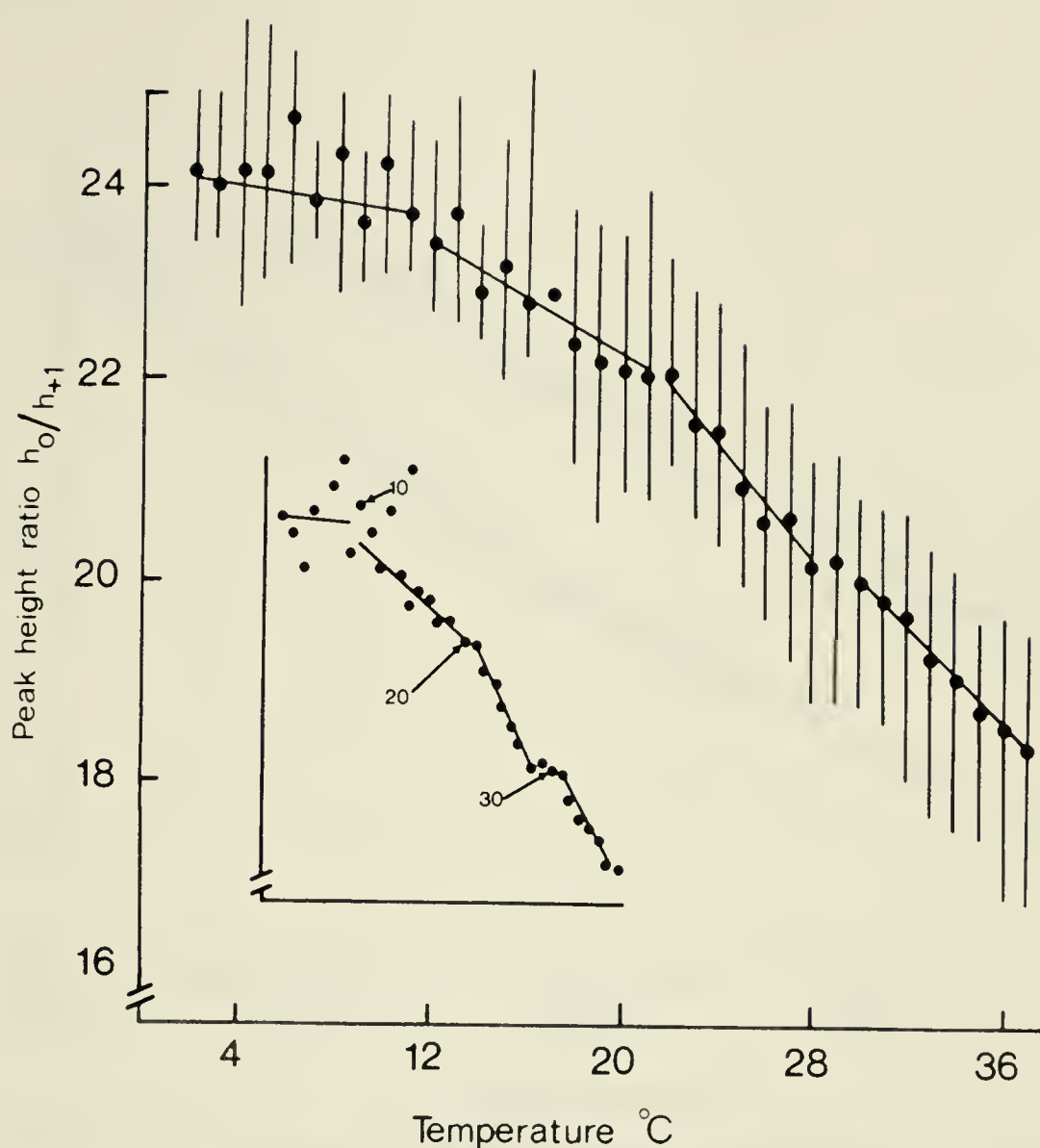


Fig. 27. A detailed study of the effects of temperature on spin labelled ox brain membranes. M 12-NSE was allowed to diffuse into membranes and was equilibrated overnight at 4°C. About 30  $\mu$ l of labelled membrane were introduced into the sample tube which was sealed. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber in intervals of 1°C, between 37°C and 2°C. The plot represents mean values of 4 determinations, with ranges. Multiple discontinuities can be seen near 10°, 20° and 30°C. These discontinuities are more marked in individual experiments and a typical plot is shown in the inset. The scatter below 10° (seen in the latter plot) may be due to "packing defects" (Lee, 1977).





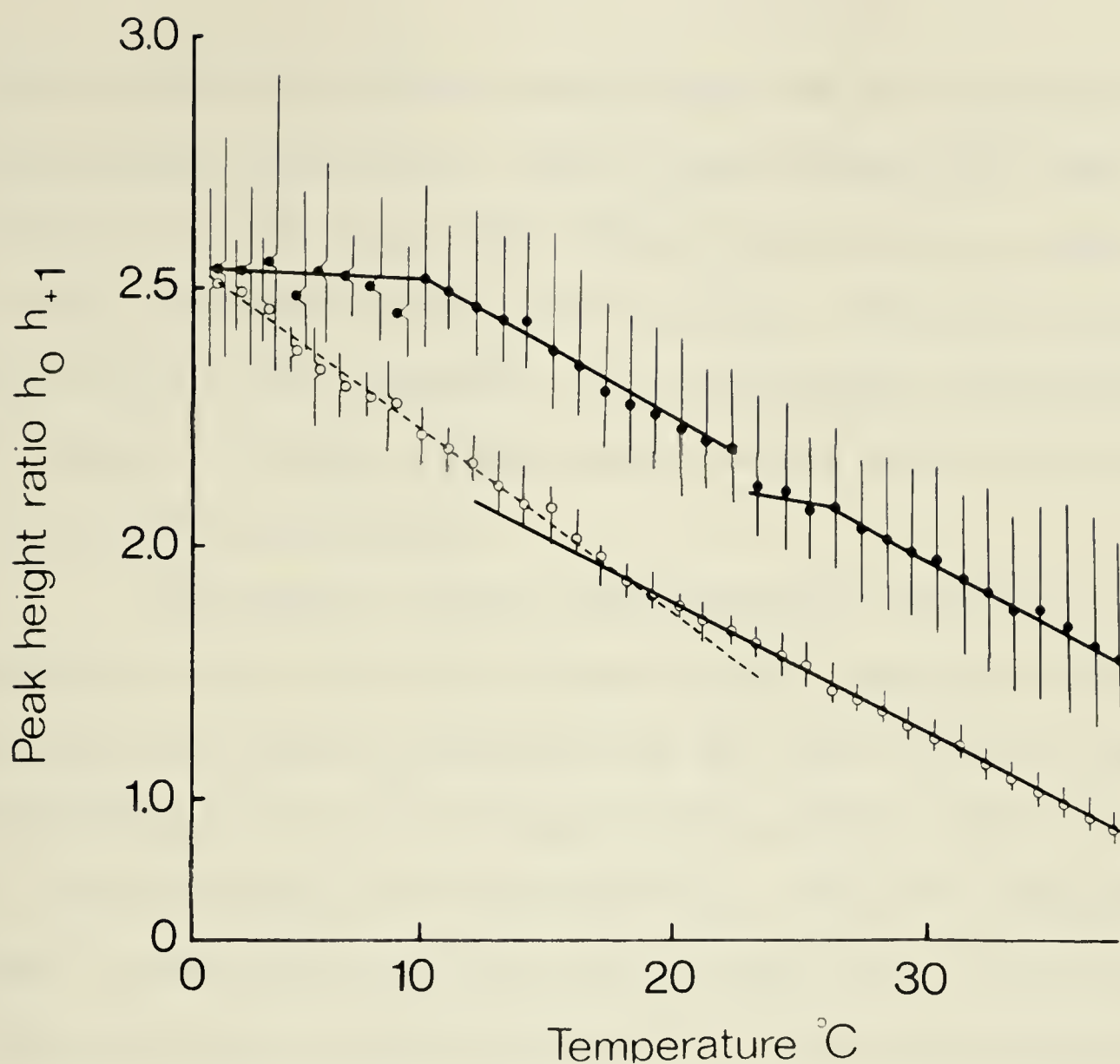


Fig. 28. A detailed study of the effects of temperature on spin labelled ox brain membranes. M 12-NSE was allowed to diffuse into membranes which had been treated with sodium deoxycholate (●) or with sodium dodecyl sulfate (○), and was equilibrated overnight at 4°C. About 30  $\mu\text{l}$  of labelled membrane was introduced into the sample tube which was sealed. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber in intervals of 1°C, between 37°C and 2°C. Both plots represent mean values of 4 determinations with ranges. Discontinuities near 10° and 26° are seen following extraction with sodium deoxycholate, whereas only a single discontinuity near 18°C occurs after extraction with sodium dodecyl sulfate.



after extraction with DOC are now different from those obtained with untreated enzyme preparations. From 37°C down to about 10°C there is no change in the slope of the line, although the presence of a step, now at about 25°C cannot be unequivocally excluded. The marked discontinuity at 10°C is still apparent. Thus on cooling DOC treated membranes, the changes in fluidity are more gradual and the marked discontinuity at about 20°C is eliminated.

This tendency towards a more homogeneous lipid matrix, is even more marked in the data obtained from SDS treated microsomes. Cooling of the labelled microsomes from 37°C down to 18°C now causes no interruption in the rate of change of probe mobility either in the form of a step or a change of slope in the plot. Below this temperature there is a small but detectable increase in the slope of the line which remains the same throughout the remainder of the temperature range that was examined. That is, there are only two states of probe mobility after treatment with SDS. This finding is essentially in agreement with the earlier results obtained from a limited number of data points. However, inspection of the present data suggests an inflection point at about 18°C rather than at 22°C as reported in the experiments described in Fig. 26.

#### 5.5 Extended study of the effects of temperature on liposomes made from lipids extracted from ox brain (Na<sup>+</sup> + K<sup>+</sup>)-ATPase.

From the experiments described above it is apparent that treatment of the membrane enzyme preparations with either DOC or SDS alters the response of the membrane lipids to changes in temperature. In order to further examine this aspect, the ESR method was applied to a



study of total lipid extracts of these preparations. Liposomes were made from these total lipid extracts and labelled with M 12-NSE. For comparison spin labelled liposomes were also made from ox brain phosphatidyl serine, a phospholipid that has been strongly implicated in the modulation of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity and temperature dependence (Schwartz *et al.*, 1975; Kimelberg, 1976).

The results are shown in Fig. 29 where it can be seen that there is a gradual increase in peak height ratio as the temperature is lowered. For both, the lipids of untreated microsomes and for liposomes made from ox brain phosphatidyl serine, this variation with temperature is readily described by two straight lines having significantly different slopes above and below intersection points at 20°C and 16°C respectively. With liposomes made from total lipid extracts of detergent treated microsomes, there is much less difference in the slopes of the lines above and below an inflection point at 20°C.

Of even more importance is the observation that the multiple discontinuities of untreated microsomes are reduced to a single inflection point in liposomes made from extracts of these preparations. Similarly the liposomes made from lipid extracts of detergent treated microsomes also differ from their parent preparations. The loss of these inflection points, strongly suggests a powerful co-operative effect by the enzyme protein on the physical behaviour of the lipid bilayer. More evidence for this co-operative effect is presented in the following section.

From the studies of the effects of temperature on spin labelled  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enriched membranes, the following summary of the





Fig. 29. A detailed study of the effects of temperature on spin labelled liposomes. M 12-NSE was allowed to diffuse into liposomes made from lipid extracts, during incubation for 10 min at 37°C. About 30 $\mu$ l of labelled liposomes were introduced into the sample tube which was sealed. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber in intervals of 1°C, between 37°C and 2°C. The results obtained from lipid extracts of membranes treated with sodium deoxycholate ( $\Delta$ ) and sodium dodecyl sulfate ( $\blacktriangle$ ) were not significantly different and showed a single discontinuity near 20°C. For comparison the results with a commercial preparation of ox brain phosphatidylserine (o) and from lipid extracts of untreated ox brain membranes ( $\bullet$ ) are given below. Single discontinuities can be seen near 16°C and 20°C respectively. The results are the mean values of duplicate determinations.



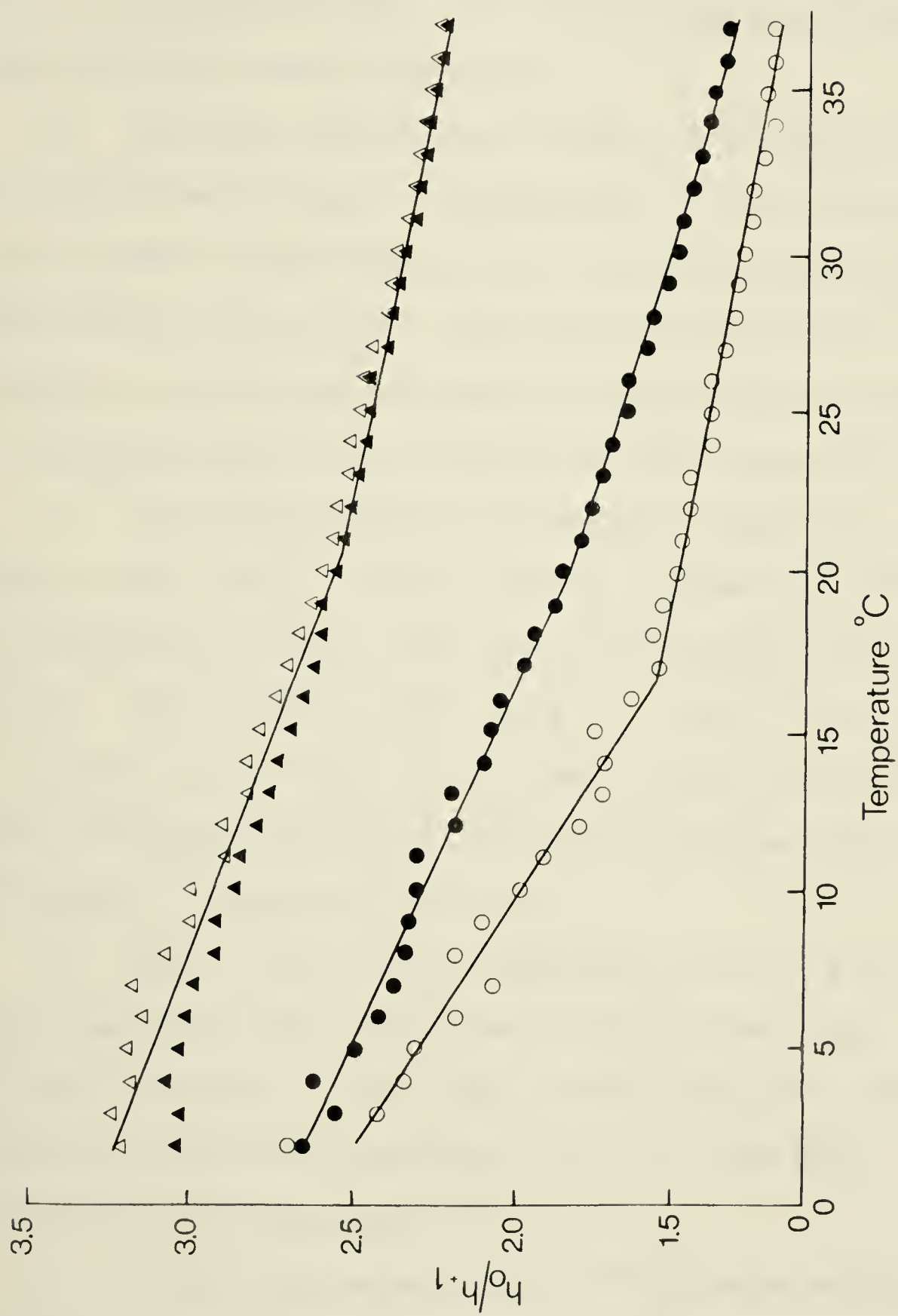


Fig. 29. Legend on separate page.



observations can be presented.

a) M 12-NSE at U sites report a sensitivity to temperature of the membrane which is apparently NOT accompanied by any thermal transitions of the membrane lipids. This result is sustained by membranes before and after extraction with DOC.

b) At R sites the spin label reports large changes in fluidity as the membrane is cooled. A limited study of 9 temperature points, when presented in the Arrhenius form, results in a typical plot with a discontinuity at about 22°C. Untreated membranes and those treated with DOC or with SDS are not markedly different either with respect to the inflection point or the slopes of the lines above and below T<sub>c</sub>.

c) An extended study of the temperature sensitivity of R sites suggests three unique temperature points, at about 30°, 20° and 10°C, when analysed as a simple linear plot of peak height ratios versus temperature. Between 28°C and 30°C there is a step, but the rate of change of fluidity remains linear. At 20°C and 10°C the fluidity changes register discontinuities; as the temperature is reduced, the rate of loss of fluidity is progressively decreased.

d) Results from extended temperature studies of R sites on DOC-treated membranes differ from those of untreated membranes. The rate of change of fluidity is now linear over the range 37° to 10°C, with a marked decrease in this parameter below this temperature. The possibility of a step at about 25°C also exists.

e) Extended temperature studies of SDS-treated membranes differ from those of untreated membranes as well as those of DOC-treated membranes.





A single discontinuity at 18°C is observed. Above and below this temperature the change in fluidity is linear with the more rapid change manifested below 18°C.

f) Finally the results from extended temperature studies of liposomes made from lipid extracts of these three types of enzyme preparations are practically identical. There is a single discontinuity at 20°C, and the change in the fluidity of the membrane is more rapid below this temperature. There is a lack of compliance between sensitivity to temperature of the liposomes and the parent microsomes.

#### 6. Extended study of the effects of temperature on the hydrolytic activity of ox brain ( $\text{Na}^+ + \text{K}^+$ )-ATPase.

The detailed ESR studies which have been described so far, have revealed hitherto undefined thermal characteristics in the behaviour of ( $\text{Na}^+ + \text{K}^+$ )-ATPase membranes; thus it was essential to re-examine the hydrolytic activity of the enzyme in the same detail. This was achieved by determining changes in ouabain-inhibitable enzyme activity at intervals of 1°C between 6°C and 37°C. Experiments with both detergent treated and untreated preparations were carried out. Fig. 30 shows Arrhenius plots of the data obtained from these experiments. In general the data from all three types of enzyme preparations are remarkably similar; in no instance can they be fitted adequately by a single straight line as there is a noticeable discontinuity at about 18°C.

The computer analysis for a two line fit was used to determine the apparent activation energies for the hydrolytic activity of the enzyme, above and below the critical temperature. The values of about 14 kcal/mole above, and about 30 kcal/mole below  $T_c$  are very similar



Fig. 30. A detailed study of the effects of temperature on enzyme activity before and after detergent extraction. The ( $\text{Na}^+ + \text{K}^+$ )-ATPase activity of untreated ox brain membranes ( $\blacktriangle \blacktriangle \blacktriangle$ ), and after extraction of the membranes with sodium deoxycholate ( $\circ \circ \circ$ ), or with sodium dodecyl sulfate ( $\bullet \bullet \bullet$ ), was determined by a continuous assay procedure. For clarity the rates of enzyme activity which are given as  $\mu\text{moles Pi/mg protein/hr}$  have been offset for the different preparations. At  $37^\circ\text{C}$  the specific activities of the preparations were 44, 125 and 95  $\mu\text{moles Pi/mg protein/hr}$  for the untreated, the sodium deoxycholate treated and the sodium dodecyl sulfate treated ox brain membranes respectively. Multiple discontinuities are apparent in all preparations.



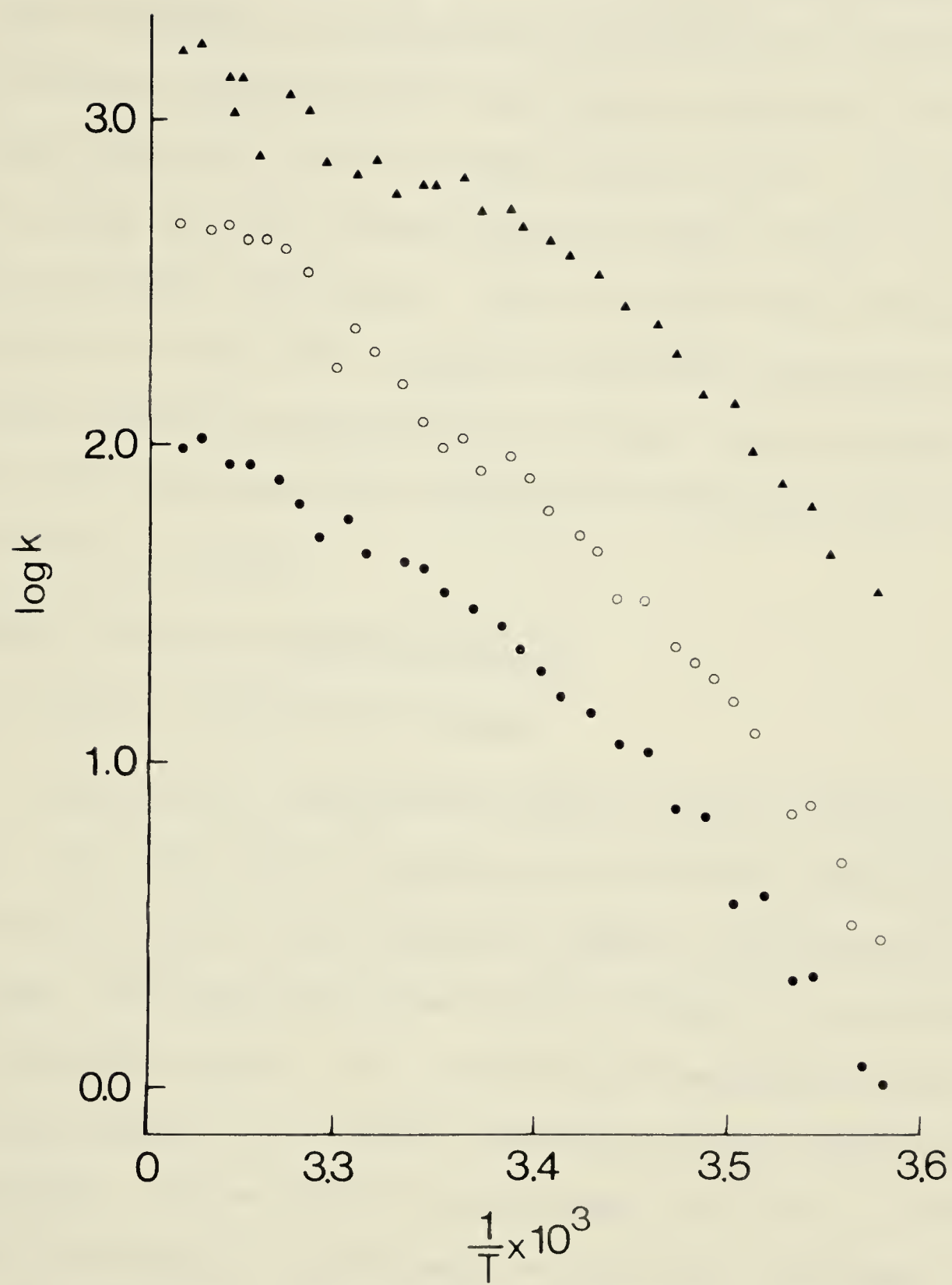


Fig. 30. Legend on separate page.





to the values obtained previously in the more limited studies carried out earlier, the results of which were given in Table 3, p. 75.

However, it seems very likely that other discontinuities which might correspond to the temperature characteristics observed in the ESR studies, also exist in this data, but are obscured by the method of analysis which only permits a single inflection point. When examined from this point of view all three plots can be shown to depart from linearity at several temperatures. There is a major step at 30°C, and a discontinuity at about 18°C. Below 6°C the rate of hydrolysis of ATP is markedly reduced and cannot be easily measured, due to technical limitations in the instrumentation; changes at low temperatures are therefore difficult to describe.

## 7. Spectral changes and membrane fluidity

### 7.1 Synthetic phospholipid bilayers.

The cooling of phospholipid bilayers leads to a decrease in fluidity. If a spin label is incorporated in the bilayer, then the reduced fluidity causes a loss of motional freedom of the nitroxyl reporter group. This effect was demonstrated in Chapter II, section 4.6, p. 56 using DMPC liposomes. Fig. 31 shows an illustration of an Arrhenius plot obtained from a typical experiment with spin labelled synthetic bilayers. As the bilayer is cooled there is at first a slow loss of fluidity, followed by a rapid change and again by a slow change. The rapid change in fluidity (the steep slope) reflects the transition of the lipids in the bilayer from a fluid phase to a gel phase, while the two discontinuities are the beginning and the end of this process (Lee, 1977). These changes can be interpreted to some degree at the molecular



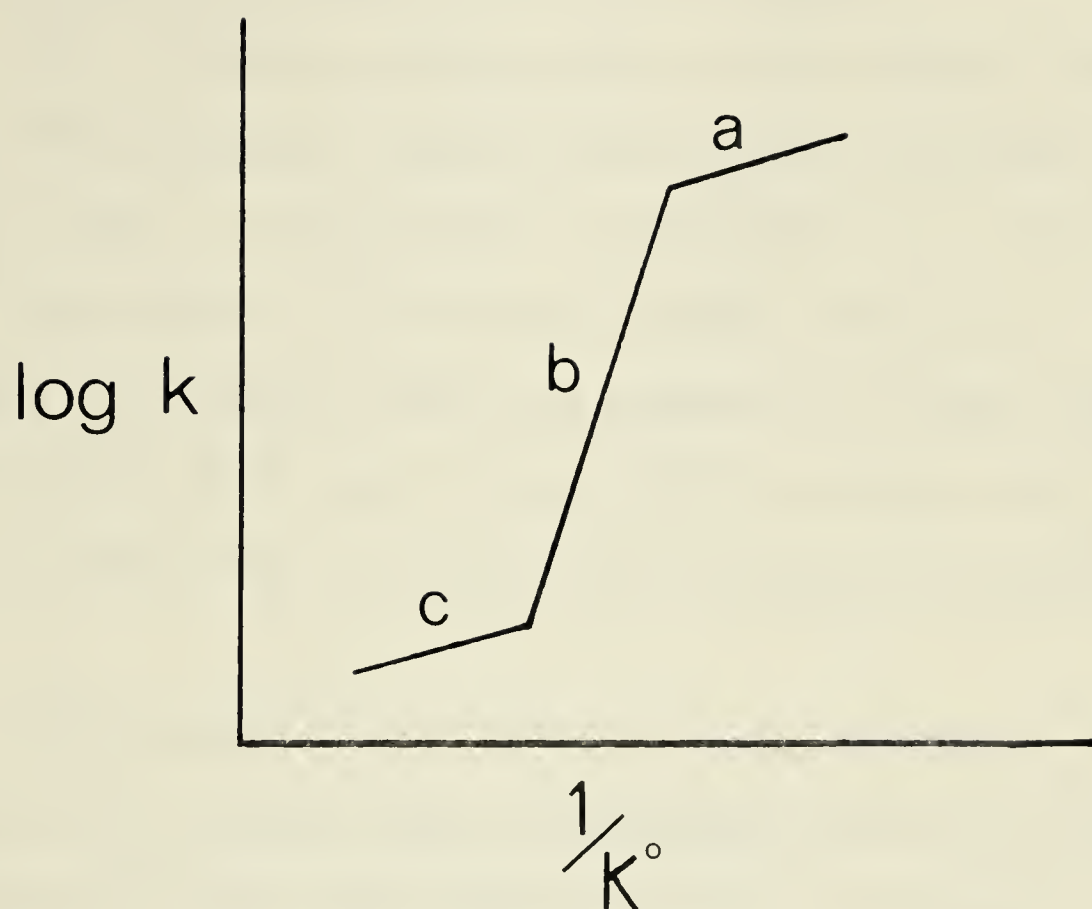


Fig. 31. Illustration of a typical Arrhenius plot of data obtained with spin labelled bilayers. An experimentally derived plot of this type can be found in Sackmann E., Trauble H., Hans-Joachim G., and Overath P., (1973) *Biochemistry*, 12, 5360.



level, but such a discussion would be inappropriate to this section, and will be found in a later section of this thesis.

Fig. 32 shows an Arrhenius plot of data obtained from spectral changes of DMPC liposomes labelled with M 12-NSE as they were cooled from 37°C to 18°C. As the bilayer is cooled there is at first a slow loss of fluidity which is followed by a sudden acceleration. Thus it is apparent that M 12-NSE reports the beginning of a phase transition in DMPC liposomes which occurs at about 21°C; the end of this phase could not be determined within the limited temperature range that was studied.

From the data in Fig. 32 the following observation is made. "The cooling of a bilayer leads to changes which occur at a slow rate before the onset of the phase transition which is followed by a fast rate of change during the phase transition". In the context of the spin label, these changes are manifested as a slow rate of decrease in motional freedom prior to the phase transition, followed by a faster rate during the phase transition. In the section that follows, this sequence of change in the motional freedom of a spin label, has been examined for ox brain microsomes labelled with M 12-NSE.

## 7.2 Spectral changes and membrane fluidity in ox brain microsomes.

The effects of temperature were first established for the enzyme activity of ox brain microsomes enriched with  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , before and after treatment with SDS. The results are shown in Fig. 33. The plot is typical of this enzyme with two distinct slopes representing different energies of activation above and below a critical temperature,  $T_c$ , such that the enzyme function is less efficient in the lower tempera-





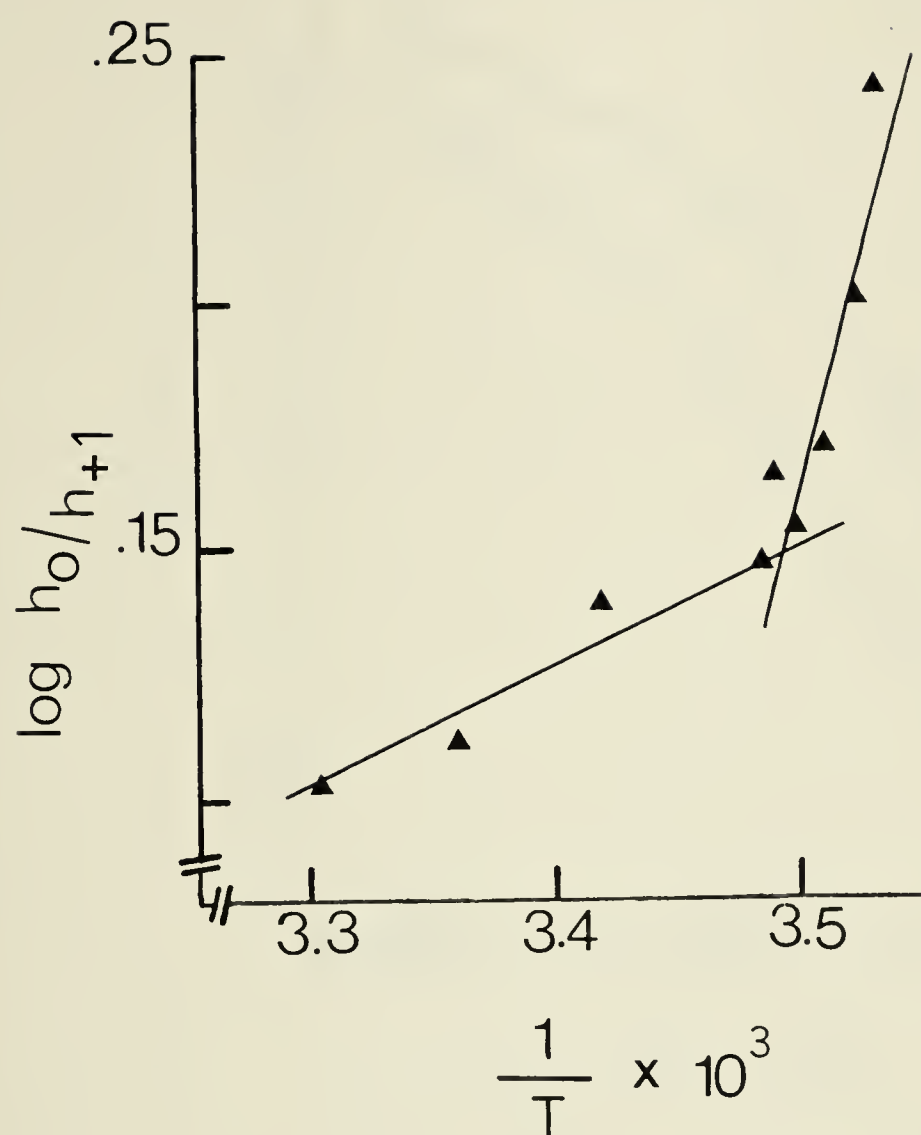


Fig. 32. Arrhenius plot of data obtained from M 12-NSE labelled DMPC liposomes. M 12-NSE was allowed to diffuse into DMPC liposomes which were then sampled for spectroscopic analysis. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber between 37°C and 18°C. As the bilayer is cooled there is at first a slow loss of fluidity followed by a sudden acceleration of the changes in fluidity below about 20°C.



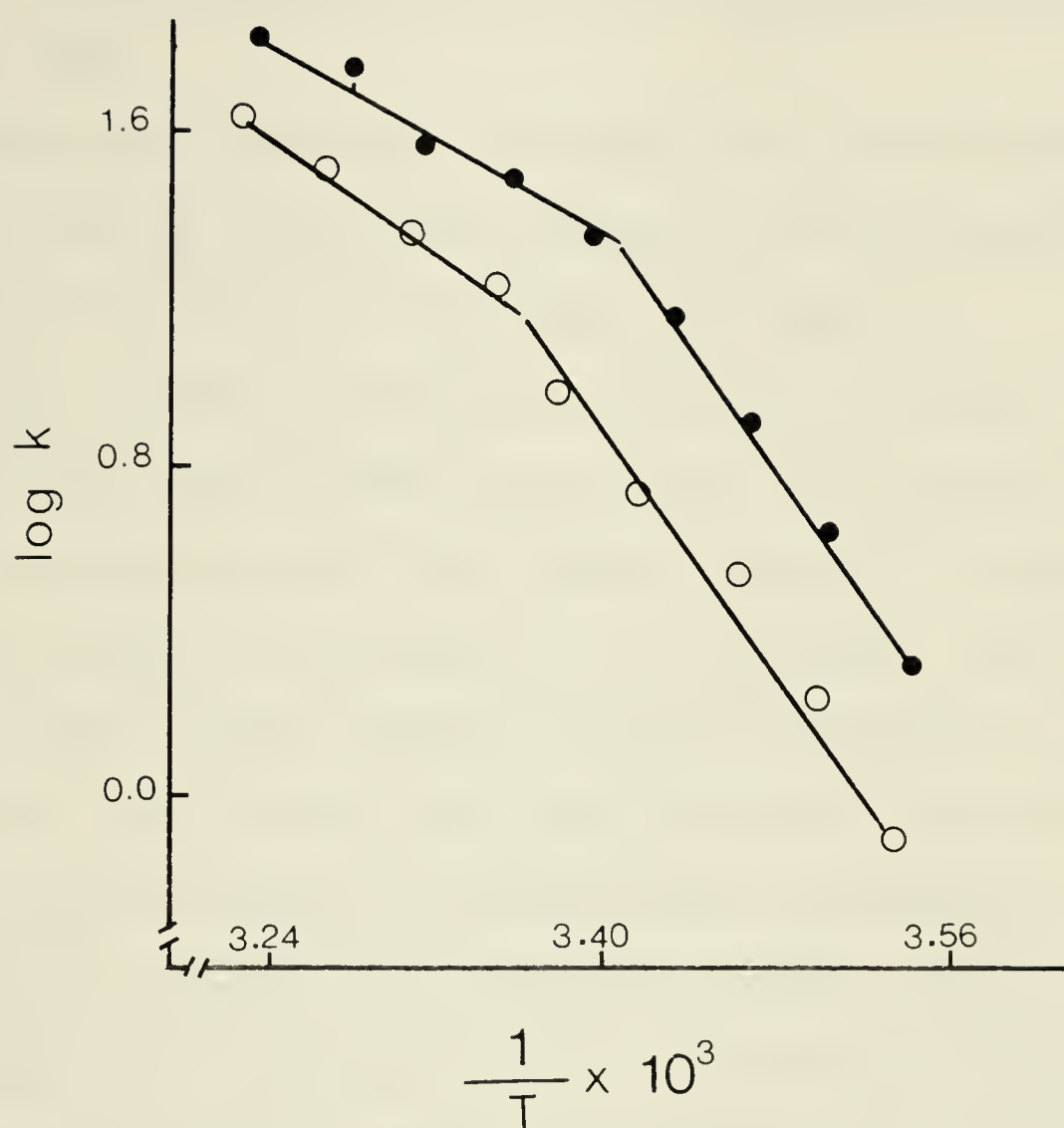


Fig. 33. Arrhenius plots of data obtained with  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enriched ox brain membranes, before and after extraction with sodium dodecyl sulfate. The  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity of untreated ox brain membranes (O) and after extraction of the membranes with sodium dodecyl sulfate (●) was determined by a continuous assay procedure. Qualitatively sodium dodecyl sulfate extraction does not alter the biphasic thermal characteristics of this enzyme.



ture range; this information has often been demonstrated before (Charnock, 1978).

It is apparent that extraction with SDS does not alter the temperature sensitivity, despite the fact that detergent treatment markedly increases the specific activity of this enzyme (see appendix V).

Fig. 34 shows an Arrhenius plot of spectral changes reported by the lipid spin probe M 12-NSE intercalated in the ox brain microsomes. The microsomes were either cooled in jumps of a few degrees (saltatory cooling) or very gradually in 1°C intervals. The biphasic response is again clearly demonstrated and the value for  $T_c$  is also similar; the two slopes in these plots now reflect two distinct rates of change of molecular motion of the probe within the membrane, such that as the membrane is cooled below  $T_c$  there is a sudden decrease in the rate of change of its molecular motion. Both saltatory and gradual cooling produced identical results. ESR data were also obtained with SDS-treated enzyme preparations and are shown in Fig. 35. The two plots represent different rates of cooling. With saltatory cooling (open circles), the SDS-treated microsomes are identical to the untreated membranes. However, when SDS-treated membranes are gradually cooled (closed circles), a markedly different plot is obtained. Although the difference in slopes is now small, the biphasic nature of the plot is still apparent and the value for  $T_c$  is the same. A notable difference however is that the relative slopes of the two lines is now reversed: that is, the loss of probe mobility is more rapid below  $T_c$ . Thus it would appear that in SDS-treated membranes, the spin probe reports from two different environments.





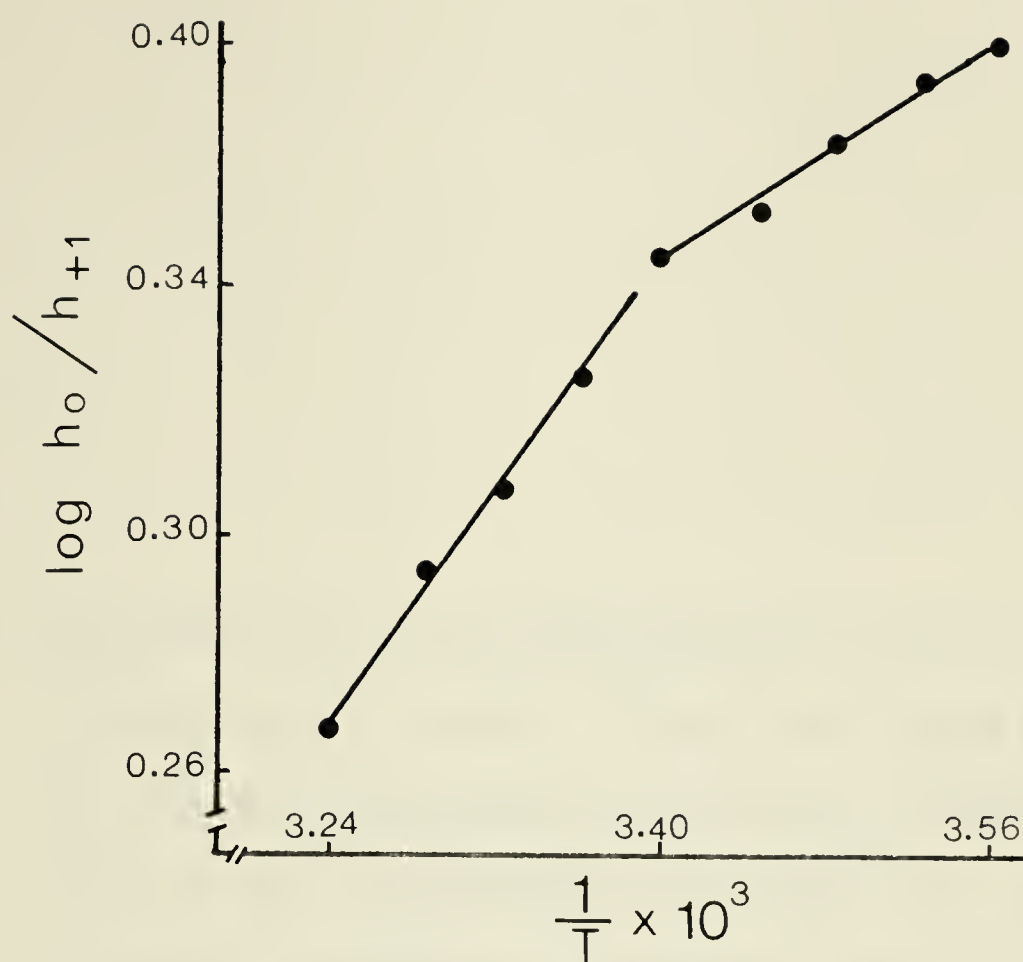


Fig. 34. Arrhenius plot of data obtained from ox brain membranes labelled with M 12-NSE. M 12-NSE was allowed to diffuse into ox brain membranes, and was equilibrated overnight at 4°C. About 30  $\mu$ l of labelled membrane were introduced into the sample tube which was sealed. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber, either in jumps of a few degrees (saltatory cooling), or gradually in 1°C intervals between 37°C and 8°C. The plot represents mean values of 4 determinations. The biphasic response of enzyme activity to temperature is duplicated by spectral changes of the probe M 12-NSE. Above  $T_c$  the changes in membrane fluidity are more rapid than below it. Saltatory or gradual cooling produced the same result.



Fig. 35. Arrhenius plot of data obtained from ox brain membranes labelled with M 12-NSE. M 12-NSE was allowed to diffuse into ox brain membranes that had been extracted with SDS, and was left to equilibrate overnight at 4°C. About 30  $\mu$ l of labelled membrane were introduced into the sample tube which was sealed. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber, either in jumps of a few degrees (○) that is saltatory cooling, or gradually in 1°C intervals (●). The plot represents mean values of 4 determinations. With saltatory cooling spectral changes of SDS treated membranes are identical to untreated membranes. With gradual cooling the sequence of the slopes is reversed; that is the change in membrane fluidity is fast below  $T_c$  and slow above it.



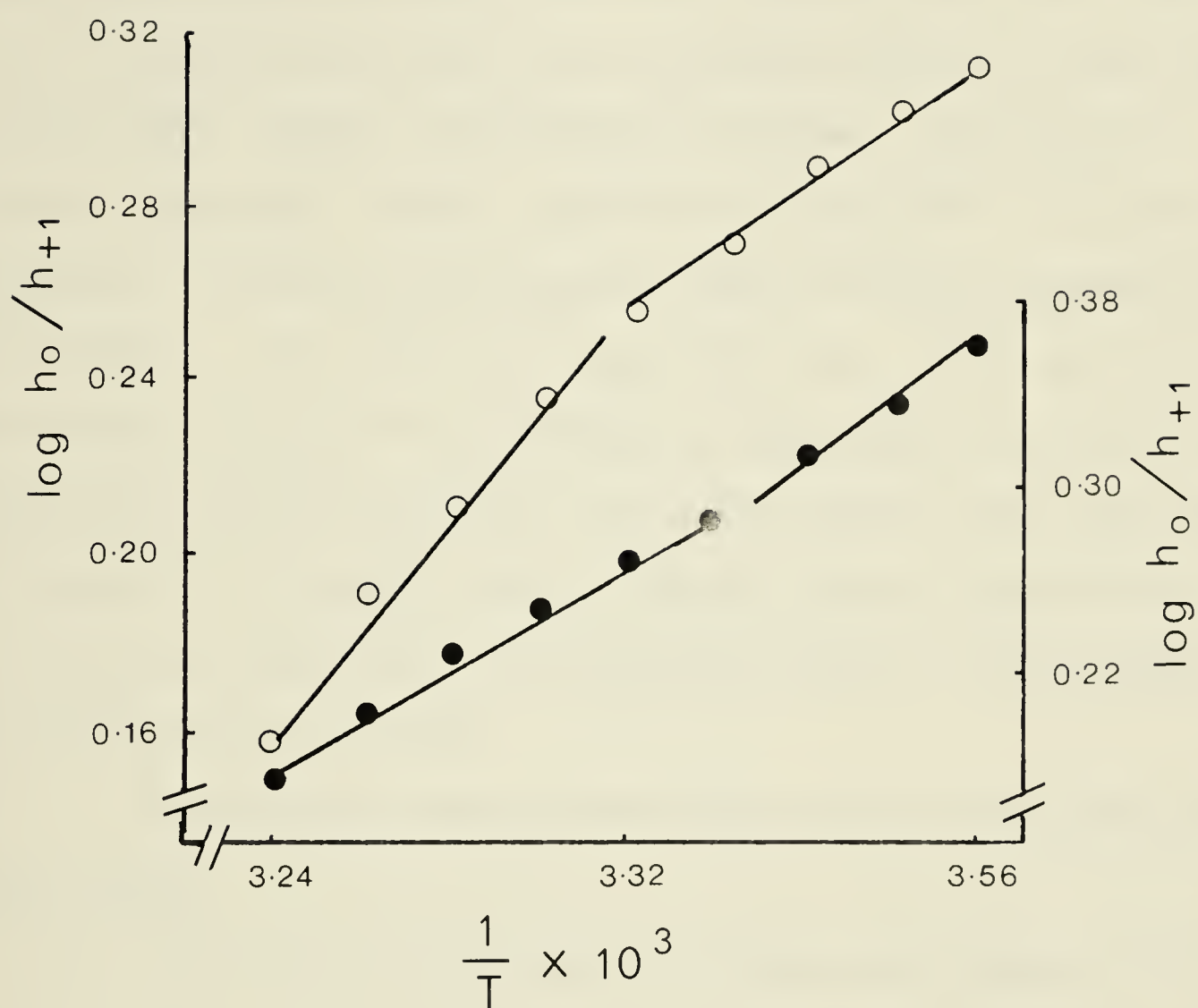


Fig. 35.

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From the results presented above, an interesting comparison can be made between microsomes and DMPC liposomes. On the one hand, untreated microsomes (irrespective of the cooling protocol), and SDS-treated microsomes subjected to saltatory cooling, both show a rapid loss of probe mobility above  $T_c$  and a slow loss below  $T_c$ ; on the other hand with DMPC liposomes the sequence is reversed. To study this 'reversal phenomenon' further, liposomes made from  $(Na^+ + K^+)$ -ATPase-free total lipid extracts of these two types of membrane preparations were examined. The results are shown in Fig. 36. As was seen with the SDS-treated microsomes, the difference in slope above and below  $T_c$  is again small; nevertheless below  $T_c$  the probe reports a more rapid rate of change in its molecular motion. Moreover, there is no difference in the effects of temperature on the liposomes whether or not the membranes had been pretreated with SDS.

Thus from the data presented above, the following observations can be listed:

- a) Purification of the enzyme by treating the membranes with SDS, does not alter its thermal sensitivity as determined by the hydrolytic activity.
- b) The changes in molecular motion of the lipid spin probe M 12-NSE qualitatively duplicate the typical temperature phenomenon associated with the hydrolytic activity of this enzyme.
- c) In spin labelled untreated microsomes, the rate of change in mobility of the probe is rapid at high temperatures, and gradual at low temperature. This result is the opposite of that found in synthetic phospholipid bilayers.
- d) When SDS-treated microsomes are spin labelled, the rate of



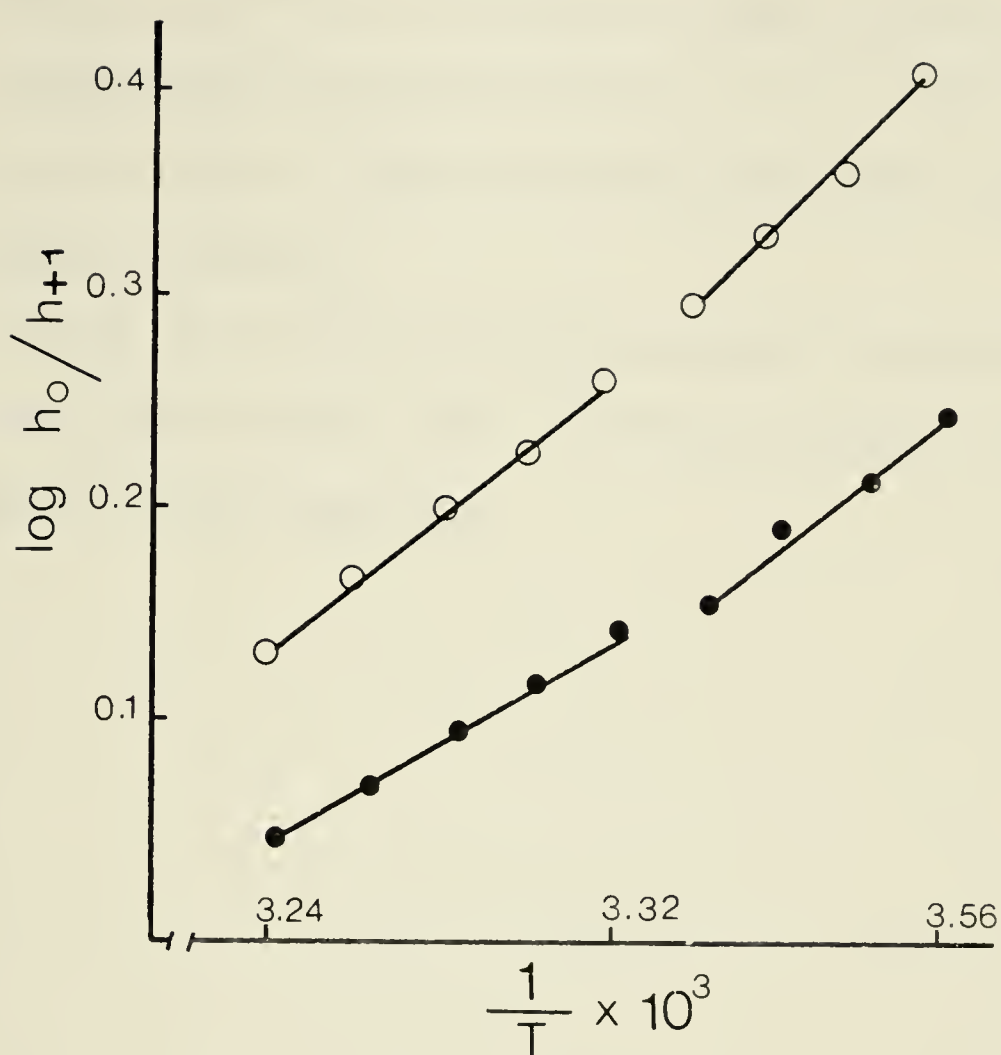


Fig. 36. Arrhenius plots of data obtained from spin labelled liposomes made from lipid extracts of ox brain membranes. M 12-NSE was allowed to diffuse into liposomes made from lipid extracts during incubation for 10 min at 37°C. About 30  $\mu$ l of labelled liposomes were introduced into the sample tube which was sealed. Temperature alteration was achieved by saltatory or by gradual cooling of 1°C intervals between 37°C and 8°C. Liposomes made from extracts of untreated membranes (O) or from SDS treated membranes (●) show no difference. Above  $T_c$  the change in membrane fluidity is greater than below it. The cooling procedure that was used also did not alter the result.



change in mobility of the probe follows the profile of untreated microsomes with saltatory cooling; with slow gradual cooling, these treated microsomes behave like synthetic phospholipid bilayers.

- e) In both the untreated and the detergent treated microsomes, the presence of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  protein is essential for this atypical behaviour.





## DISCUSSION



## 1. General

The project described in this thesis has examined two aspects of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , namely the binding of ouabain to the phosphorylated intermediate and the fluidity of the membrane in the physiological temperature range. The specific activity of the enzyme was used as an index for comparison of the two parameters mentioned above; purification of the enzyme by extraction with detergents or lipolysis with PPL-A were the treatments employed.

The specific activity of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  preparations can vary with the preparation. In addition, the preparation is usually 'contaminated' with a second ATPase which is insensitive to ouabain, the  $\text{Mg}^{++}\text{-ATPase}$ . In our laboratory, untreated enzyme preparations contain 30% or more of this second enzyme which by comparison with the distributions found in other preparations reported in the literature is relatively low. Therefore further purification is considered an essential step prior to study of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . To this end detergents are frequently employed although the precise mechanism by which they 'activate' the enzyme is not known. We have found that in SDS-treated preparations the contaminant enzyme is consistently reduced to less than 5% of the total ATPase activity. In addition to reducing the  $\text{Mg}^{++}\text{-ATPase}$  activity, detergents also serve to concentrate the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , presumably by removing extraneous proteins and lipids.

In 1964 Ohnishi and Kawamura demonstrated the involvement of lipid in the hydrolysis of ATP by  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . Since then a vast body of information has accumulated which has provided valuable information about the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  itself and also the involvement of



the membrane lipids.

The use of the effects of temperature to assess the involvement of lipids in the biochemical function of this and other enzyme systems is frequently employed, and as a consequence the Arrhenius plot has become a useful tool.

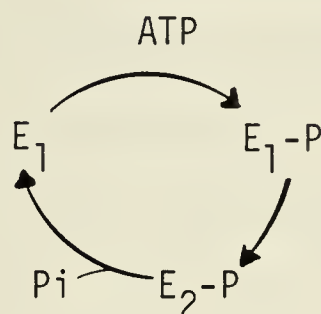
## 2. The Arrhenius plot as a biochemical tool.

At its simplest level, the Arrhenius plot offers a means whereby the effects of temperature can be related to the reaction velocity in graphic form. From such a plot the activation energy, ( $E_a$ ) can be calculated. In a number of biological reactions, the presentation of data in the Arrhenius form may result in a non-linear plot. In these situations an inflection point  $T_c$  can be derived and two energies of activation ( $E_{a_1}$  and  $E_{a_2}$ ) can be calculated above and below this point respectively. Such a situation pertains to the temperature activity relationships of the  $(Na^+ + K^+)$ -ATPase system; whereas a single inflection point has generally been accepted, the existence of more than one such temperature point has not been excluded. The importance of the Arrhenius plot to this dissertation necessitates some comment as to the implication of the  $E_{a_1}$ , the  $E_{a_2}$  and the  $T_c$  in the function of the enzyme macromolecule.

The  $(Na^+ + K^+)$ -ATPase enzyme like a number of biological molecules functions optimally at  $37^\circ C$  *in vivo*. Therefore its activation energy must necessarily be assigned the value obtained from the slope of the line above  $T_c$ , that is  $E_{a_1}$ . This energy, which for native (untreated) ox brain membranes is about 17 kcal/mole, has not yet been assigned to any specific intermediate or partial reaction of the sys-



tem. From the reaction sequence usually depicted for this enzyme (p. 9), three steps could conceivably qualify as the energy limiting events and these are shown below:



The phosphate bond of ATP is known to be a high energy bond but the activation energy for  $E_1$ -P formation has yet to be determined. In the reaction sequence of the  $(Na^+ + K^+)$ -ATPase, this step is not thought to be the energy limiting step. The conversion of the  $E_1$ -P to the  $E_2$ -P intermediate necessitates a conformational change (Post *et al.*, 1969; Post *et al.*, 1972) by the protein macromolecule. According to Alber's group (Fahn *et al.*, 1966a; Fahn *et al.*, 1966b) the  $E_2$ -P form of the phosphointermediate represents a lower energy state than its predecessor  $E_1$ -P. This does not however exclude it from being the energy limiting step. The energy necessary for driving one conformation of a protein to another, could be energy limiting and still result in a product of lower energy.

The ultimate conversion of  $E_2$ -P back to  $E_1$  and inorganic phosphate has not been examined extensively. The work in this area has been reviewed by Glynn and Karlish (1975). Although this reaction step is thought to occur spontaneously in the presence of water, there is also some suggestion that it is rate limiting (Mardh and Zetterqvist,





1972). Therefore, it must remain a contender for the energy limiting step.

Below  $T_c$ , the activation energy for this enzyme is increased two-fold. One theoretical explanation for this increase is that above and below  $T_c$  the sequence of conformational changes of the protein macromolecule may be different. That is, at the lower temperatures, the hydrolysis of ATP occurs by a different sequence and/or type of conformational change. Therefore, it is possible that the same partial reaction may still be the energy limiting step, and indeed may require different levels of energy depending on the temperature at which the reaction is carried out.

Another consideration for which there is much experimental support is the involvement of phospholipids in the operation of this membrane bound enzyme. Since the presence of some lipid/s is an absolute requirement for ATPase function, this possibility assumes importance. It is now well established that the lipid molecules of the membrane bilayer in which the enzyme is embedded are in a liquid-crystalline state above  $T_c$  and in the solid or gel phase below it. If lipids are involved in one or more of the partial reactions of  $(Na^+ + K^+)$ -ATPase then they could directly influence the activation energy of the enzyme. That is to say, the lipid contribution to the overall energy requirements of the  $(Na^+ + K^+)$ -ATPase might be reduced below  $T_c$ . If the information from the two slopes of an Arrhenius plot reflect the involvement of lipids in the energy requirements of the reaction mechanism, then three possibilities must be considered.

- a) At temperatures below  $T_c$ , the lipids in their solid or gel state might hinder or retard the protein from attaining its



conformational forms.

- b) Conversely, at temperatures above  $T_c$  the liquid crystalline state of the lipids might facilitate this process and thus reduce the energy requirements of the reaction sequence as a whole.
- c) It is also possible that above as well as below  $T_c$  the protein and lipid components of the membrane bound enzyme obligatorily cooperate to achieve the process of ATP hydrolysis.

If the concept of hindering (case a) is correct then Arrhenius plots of partially delipidated enzyme preparations would show a decrease in the difference between the two activation energies.  $E_{a1}$  should remain unchanged while the value for  $E_{a2}$  should show a tendency towards approaching that of  $E_{a1}$ . If facilitation (case b) is the explanation, then the situation would be reversed; the value for  $E_{a1}$  would be altered towards a value nearer  $E_{a2}$ . Since the activation energy derived from linear plots is usually a value intermediate to  $E_{a1}$  and  $E_{a2}$  these two hypotheses do not seem to be likely. Therefore the third postulate, namely a co-operative protein-lipid effect must assume predominance in an interpretation of lipid involvement.

In the chapter on results section 7.1 a typical Arrhenius plot of a synthetic bilayer was illustrated (Fig. 31). From such a plot the temperature of the phase transition is readily obtained from the midpoint of the steep slope. If two lipids with differing transition temperatures are mixed then the transition temperature of the mixed bilayer is now a value intermediate to the individual values of the constituents. This is, the slope is less steep. In biological membranes, the phospholipids are usually a heterogeneous mixture with varying head groups, chain





length and chain saturation. Therefore, true phase transitions may not occur. Nevertheless biophysical techniques, such as those with spin probes (Grisham and Barnett, 1973) or fluorescent probes (Charnock and Bashford, 1975), have clearly established discontinuities in slopes obtained from temperature induced changes in the mobility of reporter molecules. These discontinuities may represent separations leading to the formation of clusters, or they may represent phase transitions of clusters or domains. For this reason in this dissertation  $T_c$  was defined as the temperature of the inflection or discontinuity and not the phase transition temperature.

### 3. Hydrolytic Activity of $(Na^+ + K^+)$ -ATPase

The early studies of the effects of temperature on  $(Na^+ + K^+)$ -ATPase preparations from rabbit kidney cortex show that the enzyme displays the typical characteristics in that it yields a non-linear Arrhenius plot with distinct activation energies above and below a critical temperature. That is to say for the hydrolysis of ATP, this enzyme from rabbit tissue requires about 18 kcal/mole above and about 39 kcal/mole below a unique temperature, which from these studies is about 18°C. Furthermore, if the enzyme is treated with detergents such as DOC or Nonidet-P 40, these parameters do not change although the specific activity is markedly increased.

The two-fold increase in activation energy may hold information of some significance. In a recent review on 'membrane lipid phase-transitions', Charnock (1978) listed the activation energies for  $(Na^+ + K^+)$ -ATPase obtained from homeotherms, poikilotherms and hibernators. Although the values for  $E_a$  from these preparations varied by 71%





(from 12.5 to 21.4 kcal/mole) and the corresponding variation for  $Ea_2$  was 90% (from 23.7 to 45.1 kcal/mole), the ratio of these two energy requirements was consistently close to two-fold, with a mean ratio for  $Ea_2:Ea_1$  of 2.1 and a range from 1.8 to 2.3.

Our later work with  $(Na^+ + K^+)$ -ATPase from ox brain shows that the thermal phenomena are very similar to those of the rabbit enzyme. The mean value for  $Ea_1$  for untreated as well as detergent treated membranes was 17.0 kcal/mole, and for  $Ea_2$  was 38.0 kcal/mole. Once again the activation energy is increased about two-fold below  $T_c$ , the ratio of  $Ea_2:Ea_1$  being 2.2.

The results from studies of the hydrolytic activity of ox brain membranes were summarized in Table 3. Although the characteristic thermal phenomena are common to the different preparations and treatments (except PPL-A), some variation is apparent in the activation energies, both above (13.6 to 21.8 kcal/mole) as well as below (29.8 to 49.2 kcal/mole)  $T_c$ . By comparison the temperature of the inflection was less varied (16.6 to 20.9°C). It is not possible to explain these differences from what is currently known about this membrane bound enzyme. At the most, some tendencies can be pointed out.

For example,  $(Na^+ + K^+)$ -ATPase prepared from membranes that were homogenized with the polytron were usually more active than those prepared after disruption with the glass-teflon homogenizer. This increase in specific activity was accompanied by values for  $Ea_1$  and  $Ea_2$  that suggested greater fluidity. This tendency was also apparent with membranes that had been extracted with detergent. This mere hint makes a meagre contribution towards understanding the involvement of the lipids in  $(Na^+ + K^+)$ -ATPase function. Even SDS treatment which frequent-



ly yielded preparations with a 10-fold increase in specific activity, did not alter the biphasic nature of the Arrhenius plot or the inflection temperature. In fact the sharpest delineation of the discontinuous Arrhenius plot was often found following extraction with this detergent. Thus, although it is a reasonable assumption that tightly bound lipids must increase in parallel with the catalytic activity, our studies of the effects of temperature on the hydrolysis of ATP do not provide unequivocal evidence to support this assumption. In this regard it should be recalled that Wheeler *et al* (1975) were unable to show any marked change in phospholipid composition despite a 50-fold increase in specific activity of the membrane preparation after deoxycholate treatment and density-gradient centrifugation. Perhaps a better understanding is restricted by one or more of the limitations listed below:

- a) The method used for determining the effects of temperature lacks the necessary precision.
- b) The lipids are being concentrated in domains as a consequence of purification and their relative concentration is too small to have any marked effect on the thermal characteristics of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . The work from Wheeler's laboratory (1975) would certainly support this assumption.
- c) The lipids being concentrated along with the protein macromolecule are not singularly responsible for the thermal phenomena associated with this enzyme, but jointly with the protein as well as the bulk lipid.
- d) It is possible that the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  is capable of accommodating a degree of change in its membrane environment



without consequence to its function. That is to say, changes may well occur in the composition of the membrane lipids on purification, but the protein-lipid interaction being dynamic in nature can adjust to accommodate the alteration.

The effects of temperature on the initial rate of [ $^3\text{H}$ ]-ouabain binding to the enzyme revealed that like the ouabain-sensitive hydrolysis of ATP, an Arrhenius plot of the temperature-rate relationship was biphasic. However the difference in the apparent energies of activation above and below the inflection point was noticeably decreased. With preparations made by homogenization of the membranes with the polytron disintegrator, this tendency towards linearity was even more marked than when homogenization was carried out in a teflon-glass apparatus although the reason for this is not clear. If it is a consequence of the method of preparation then a loss of some lipid/s must result from the increased disruption, implying that the lipids associated with the binding site for ouabain are easily dislodged or altered in some way. This concept is supported by the fact that detergent treatments (DOC and SDS) which are without effect on the thermal sensitivity of the enzyme, readily abolish the effect of temperature on the initial rates of ouabain binding.

In addition, when untreated membranes prepared by disruption with a glass-teflon homogenizer were examined, non-linear Arrhenius plots were obtained whether or not the membranes were first extracted with DOC at 4°C. These observations do not agree with those of Siegel and Josephson (1972) or of Wallick and Schwartz (1974). However the





experiments by Siegel and Josephson (1972) were conducted after incubation for 15 minutes, which according to our very detailed study would only yield values for initial rates at the very low incubation temperatures in the range we examined or equilibrium levels only at the higher temperatures. In the report by Wallick and Schwartz (1974) the data points are too few to permit an analysis by the Bogartz technique used in this work (Cook and Charnock, 1978, in press); nevertheless on close inspection their published results might well be described as non-linear! Both these apparent discrepancies are discussed in greater detail in our recent publication on this subject (Charnock, Simonson and Almeida, 1977).

The rate of [ $^3\text{H}$ ]-ouabain binding to PPL-A treated membranes yielded a linear Arrhenius plot. Interestingly, the addition of PS did not restore the non-linearity. This result is different from that obtained by ouabain inhibition of membrane ( $\text{Na}^+ + \text{K}^+$ )-ATPase activity that had been subjected to lipolysis with PPL-A. In the latter experiments, PS restored the non-linearity. Thus it appears that the binding of [ $^3\text{H}$ ]-ouabain is less dependent on lipid than is the inhibition of the enzyme.

##### 5. Lipolysis with phospholipase-A

In contrast to the effects of purification by detergent treatment, PPL-A has a dramatic effect on both the specific activity as well as the thermal phenomenon of the enzyme.

With previously untreated membranes, PPL-A first causes an increase in specific activity followed by a progressive decline to the untreated level. In the case of detergent treated membranes, which





have a higher starting specific activity, PPL-A did not cause any further increase in this index. Instead, the loss of activity on exposure to PPL-A with this agent was immediate, and progressed to the same level as that of the treated membranes. This distinction, namely the increase in catalytic activity by an agent which acts on the lipid and not on the protein (Hokin and Hexum, 1972), is not easily explained without invoking protein-lipid interactions. It is possible that the alteration of lipids in untreated membranes leads to a loss of non-enzymatic protein which in turn would result in larger numbers being obtained for specific activity without any true increase in the number of catalytic sites. In the case of detergent treated membranes, this loss of non-enzymatic protein would occur during the purification procedure. In other words, the distinction between untreated and treated membranes might only be a reflection of the inability of the assay procedure for protein content to distinguish between enzymatic and non-enzymatic protein molecules.

Alternatively, the increased activity could result from the exposure of latent sites by the lipase treatment. This latter suggestion was initially made for detergent activation of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  by Jorgensen and Skou (1971) and has since been supported by several other groups including our own laboratory (Charnock *et al.*, 1977).

When treated or untreated membranes were exposed to the action of PPL-A, a proportion of the catalytic sites were found to be resistant to the effects of membrane lipolysis. This observation is also not easily explained. Again it is possible that there are two distinct populations of catalytic units, the lipase resistant population being



considerably smaller in number. However, if it is recalled that the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  has an absolute requirement for lipid (Roelofsen *et al.*, 1971), then the lipase resistant population presumably has a singularly different lipid requirement. This interpretation would be supported by the frequently demonstrated result, the Arrhenius plots of this lipase treated enzyme are linear; the results of reactivation experiments with PS however, would not (Charnock *et al.*, 1975).

Temperature studies showed that delipidation of ox brain microsomes with PPL-A generated a marked change in the values of the two activation energies.  $E_{a1}$  shows an increase while  $E_{a2}$  decreases. This is unlike the tendency towards a more fluid membrane that was observed with detergent treatment. Thus whether these catalytic sites are new or not, the nature of lipid involvement in the catalytic activity is markedly altered as a consequence of PPL-A treatment. In addition, unlike our previous findings with  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  from rabbit kidney cortex (Charnock *et al.*, 1973, 1975), and those of Taniguchi and Iida (1972) the specific activity when presented in the Arrhenius form did not now yield a linear plot. An inflection point could be detected at 30°C and although the two activation energies were not markedly different, their slopes were inverted. From the data it is not clear why this enzyme, after PPL-A treatment would be more efficient below the transition than above it, but it is of interest that this situation has been observed in a number of other biological situations where the source of the enzyme is an organism having unusual temperature requirements (Charnock, 1978).

Our observation (Charnock *et al.*, 1975) that the effects of





temperature on the cation activation of this enzyme are not altered by delipidation with PPL-A does not support the two site concept. Therefore, the possibility must be entertained that only a single population of catalytic units exists. Simpkins and Hokin (1973) have suggested that inactivation of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  by PPL-A may be due to a conformational change in the protein resulting from destabilization due to disruption of the phospholipid bilayer. If this is so then the residual enzyme activity may arise from catalytic sites functioning sub-optimally by employing alternate phospholipids in the membrane in a sub-optimal protein-lipid interaction. An interpretation such as this is favoured by the following information:

- a) Residual catalytic units yield Arrhenius plots having a unique slope.
- b) Some workers have achieved similar linear plots by extensive delipidation with detergents.
- c) The effects of delipidation with PPL-A can be restored by PS.

## 6. Binding of ouabain to $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$

The binding of ouabain to the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  was examined in three different types of membrane preparation, namely PPL-A treated, untreated and detergent treated, and the information will be discussed in this order.

As was observed with the experiments on the specific activity, the binding of ouabain to PPL-A treated membranes showed an increase initially followed by a followed by a progressive decline. If however the membranes were first purified with DOC then this biphasic response was no longer present. In contrast however, the binding of ouabain did





not decrease in parallel with the catalytic sites, whether or not the membranes were first purified with DOC. This result is in agreement with the earlier findings of Erdmann and Schoner (1973) but not in agreement with those of Goldman and Albers (1973), making further discussion necessary.

In discussing the increase in specific activity as a consequence of PPL-A or detergent treatment, the existence of two populations of catalytic units was introduced. Since the catalytic subunit of the enzyme macromolecule carries the phosphorylation site (as was documented in the introduction of this thesis) and ouabain binds to the  $E_2$ -P intermediate, it follows that the catalytic subunit also carries the ouabain binding site (Kyte, 1972a,b). Thus the concept of two populations of catalytic units must be extended to two populations of ouabain binding sites. As the catalytic sites increase after treatment with PPL-A or purification with DOC (exposure of latent catalytic subunits) so do the ouabain binding sites. However, further examination showed some discrepancies in this correlation, and this information is discussed below. As was presented in the chapter on Results, the experimental procedures enabled a study of the rate of ouabain binding as well as the amount of drug that was bound under equilibrium conditions. Thus, using the definitions of Erdmann and Schoner (1973, 1974) these experiments permitted a determination of both the affinity and also the number of ouabain binding sites. A summary of the data illustrating the effects of PPL-A on the specific activity and also on the affinity and number of ouabain binding sites was shown in Table 6 p.99. PPL-A decreased the specific activity to 15% of the pre-



treated control. In contrast the affinity of ouabain binding was reduced to 53% while the number of sites did not fall below 73%. So the catalytic activity exhibits a markedly greater sensitivity to the lipolytic effects of PPL-A than does the affinity or the number of ouabain binding sites. Furthermore, the affinity and the number of ouabain binding sites also exhibit distinct sensitivities to this lipolytic agent.

Therefore the credibility of the 'two population concept' that was described above is again called into question: if PPL-A causes destruction of a whole population of catalytic units, then the same effect is not observed either for the affinity or the number of drug receptors. That is to say, some of the catalytic units, despite having lost their catalytic activity still retain their capacity for binding ouabain. Nevertheless the observation is in agreement with the earlier work of Taniguchi and Iida (1973) and the recent conclusions of Hansen (1976) that two different types of ouabain binding sites are available in  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enriched membranes. Let it be emphasized however, that the alternate suggestion made above, of a single population of catalytic sites would also explain the findings. According to Simpkins and Hokin (1973) these sites are conformationally modified after PPL-A treatment.

If two populations of ouabain binding sites exist, the question arises as to whether these sites also exhibit multiple affinities. The data in Fig. 8A show that the rate of ouabain binding increases linearly with the specific activity, implying no change in the characteristics of the binding sites as the specific activity of the enzyme increases. However it was pointed out that the use of protein content as a reference



point for binding studies could obscure changes in affinity. By using a ratio of two rates which represent the catalytic and the drug binding properties of the enzyme, namely Pi release and ouabain binding, use of the protein concentration was avoided. The data shown in Fig. 9 p. employ this method of presentation. The absence of a line parallel to the abscissa permits the unequivocal statement, that "the affinity of the drug for the receptor is not constant over the range of specific activities that were examined" (10 to 158  $\mu$ moles Pi/mg protein/hr). It is also definite that the more pure enzyme preparations show an apparent decrease in affinity for the drug, which is in agreement with the findings of Lane *et al* (1973). These workers reported that a highly purified preparation of canine kidney ( $\text{Na}^+ + \text{K}^+$ )-ATPase bound [ $^3\text{H}$ ]-ouabain at a slower rate than a relatively impure ( $\text{Na}^+ + \text{K}^+$ )-ATPase preparation from ox brain. However from the data presented in Fig. 9 it is not possible to categorically exclude the possibility of two distinct affinities, as opposed to a continuous gradual change.





The discussion of the 'two site' concept as it pertains to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  embraced a variety of information. In the following summary the salient features have been accumulated.

a) There are two populations of catalytic units, identifiable by activation with detergents or inactivation with PPL-A. When examined as an Arrhenius plot, the biochemical activity of these units as determined by ouabain inhibition does not differentiate between these two populations; however, after lipolysis with PPL-A, an agent which does not directly alter the structure of the protein macromolecule, some difference is apparent. In contrast, cation activation of the enzyme does not distinguish the two populations thereby recognizing only a single population. In addition, reactivation with PS after lipolysis with PPL-A also obscures or abolishes the difference.

b) There are two populations of ouabain binding sites, both of which reside on the catalytic subunit of the enzyme macromolecules. They apparently do not correspond either in number of PPL-A sensitivity to the catalytic units. The affinity of ouabain for the binding sites is not uniform. It is not known however whether the affinity has a binary distribution or whether a continuous variability exists.

Thus the 'two site' concept incorporates a number of observations, hypotheses and contradictions. In the opinion of this author a simpler model of a protein macromolecule with versatility of form and function might be a better concept. Such a model would avoid the rigidity of 'a site' and permit the advantage of a dynamic protein in a dynamic bilayer, at least until further information about this macromolecule becomes available. Therefore further investigation into the



properties of the single macromolecule would be more profitable than the demographic interpretation discussed above.

In the introduction, two questions were posed as being of importance to the study of ouabain binding to the  $E_2$ -P intermediate. One of these is repeated below:

"Will the pharmacological actions of these drugs, namely the therapeutic and the toxic effects, be better understood by examining the binding of ouabain to the enzyme?"

Evidence has been presented that the affinity of the drug for the receptor decreases with purification of the enzyme. Furthermore, this effect might be consequent to the sequestration of specific lipids associated with the enzyme (as was shown from the ESR experiments). Taken together these observations provide a basis for future research into the toxicity of the cardiac glycosides. Can it be that toxicity, an important clinical aspect of these drugs bears some relationship to altered membrane lipids in the vicinity of the receptor?"

## 7. Electron spin resonance studies.

Although biophysical techniques such as fluorescence and ESR spectroscopy are extensively used to follow possible changes in the physical properties of lipids within biological membranes, it is not yet possible to precisely locate the reporter molecule. Thus spin labels may report from specific domains, from the bulk lipid, or from both (Jost *et al.*, 1973a,b). In addition, the spin probes may also dissolve in the aqueous solution and give rise to "liquid lines" (Giotto *et al.*, 1973; Butler *et al.*, 1974). The spin labelling method



described in this dissertation permits the direct labelling of biological membranes by diffusion of the spin probe M 12-NSE from the surface of the glass sample tube. Any residual label on the surface of the glass was shown to be of no consequence in the experiments that were conducted. It was also shown that the esterified form of this stearic acid spin label had a very low solubility in aqueous media making it possible to avoid any contribution to the spectrum from labels in solution.

This label readily diffused into liposomes made from DMPC, and these liposomes were used to show the effects of temperature on such bilayers, as well as to define the most appropriate method of spectral quantification. The results showed that M 12-NSE was an appropriate label for the study of fluidity changes in biological membranes. However when ox brain microsomes are spin labelled with M 12-NSE a variety of spectra may result. At high probe:membrane ratios the spectrum is isotropic, resembling that of a nitroxide reporter group tumbling freely in solution. At low probe:membrane ratios the spectrum becomes broadened and now resembles that of spin labelled liposomes. These two limiting cases are quite distinct and are best interpreted as originating from spin probes at two independent sites, an unrestricted site, designated as U and a site of considerably more restricted motion designated as R. Both U and R sites have been characterized to some degree. Labels at U sites are rapidly reduced by ascorbic acid and report a moderate change in fluidity on cooling the bilayer. Tumbling times were calculated (Raison *et al.*, 1971) for M 12-NSE at U sites, and were found to be similar to those for a water soluble spin probe in 0.25 M sucrose. Thus it is possible that U sites might be located at the membrane-water







interface. Such a position would comply with the rapid reduction of the spin label by ascorbic acid, but would require a bending of the polymethylene chain as described by Cadenhead (1975). Conversely, it is also possible that the nitroxyl resides in a very fluid area of the bilayer. Typical isotropic spectra such as those obtained with labelled U sites, have been described for spin labels in the lipid core of a model bilayer in the liquid crystalline state (Seelig, 1970). A hyperfine splitting value of 16 G was reported in this work by Seelig, and it compares favourably with 15.8 G, the value obtained from data described here.

Spin labels at R sites experience a marked increase in the viscosity of their environment as the temperature is reduced. The similarity of spectra obtained with labelled R sites and DMPC liposomes is indicative of a location deep in the hydrocarbon bilayer for these binding sites, and this is further supported by the lack of effect of ascorbic acid that was observed.

If both U and R sites on a membrane sample become labelled, then a mixed spectrum results. However, some experimental control of this mixture is possible. By adjusting the ratio of the probe to the amount of enzyme, the binding of M 12-NSE can be directed predominantly to one population of sites. Thus it is possible to examine either U sites or R sites with minimal interference from the other. This controlled labelling was of particular importance in the later work which was intended to examine the effects of temperature on the fluidity of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enriched membranes.

The effect of membrane concentration on the spectrum varies from one enzyme preparation to another, and this variation can be correlated with the degree of purification of the membrane-bound



( $\text{Na}^+ + \text{K}^+$ )-ATPase. This was especially noticeable with membranes that had been extracted with SDS. High activity SDS treated membranes appeared to be homogeneously labelled at the R sites. This correlation between R sites and specific activity of ( $\text{Na}^+ + \text{K}^+$ )-ATPase suggests a location for the R sites that is associated with the protein macromolecule.

The linear response to temperature of M 12-NSE at the so-called unrestricted or U sites lends further support to the suggestion made above, that this site is inherently very fluid whether it is at or near the membrane interface or deep within the membrane core. Wherever it is located, it is not influenced by thermal transitions within the membrane lipids, when examined by M 12-NSE, and is consequently of marginal interest to this work. However labelling at this site must be accounted for when interpreting data in subsequent studies.

On the other hand, ox brain microsomes labelled with M 12-NSE at restricted or R sites are markedly influenced by thermal transitions within the membrane. In our so-called limited studies which only employed nine observations within the temperature range  $0^\circ$  to  $37^\circ$ , the spin label reports fluidity changes with temperature that closely parallel the thermal sensitivity of ( $\text{Na}^+ + \text{K}^+$ )-ATPase. These results support the general conclusions of Grisham and Barnett (1973) who used spin labels and Charnock and Bashford (1975), who employed fluorescent membrane probes.

When the R sites are examined in studies with maximum data input that we could achieve and with minimal bias in the analysis, multiple temperature dependent inflections are revealed. For example, there is evidence that three such temperature points exist in microsomal enzyme preparations which have not been subjected to extraction by detergents.





It is singularly important to this work, that similarly detailed studies of the temperature-activity relationship of the hydrolytic activity of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  also reveals multiple discontinuities. Hence it can be seen that for untreated ox brain membranes the correlation between the thermal sensitivity of the enzyme and of the spin probe at R sites is fairly good.

As detergent extraction is applied to these preparations this level of correlation between ESR data and  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity declines. For example some of the thermal effects upon the enzyme activity cannot be detected in either the DOC or in the SDS treated membranes. This is particularly noticeable in liposomes of the total lipid extracts of these preparations, all of which were devoid of multiple inflections, and could be characterized by a  $T_c$  at about  $20^\circ\text{C}$ . Liposomes from extracts of both detergent treated membranes, were also identical in their slopes above and below  $T_c$ , while the liposome preparations from extracts of untreated membranes showed the greatest overall change in fluidity with temperature, in the range  $37^\circ\text{C}$  to  $3^\circ\text{C}$ .

From the studies of the effects of temperature on spin labelled microsomes, it is apparent that the R sites report an increasing homogeneity in the membrane lipids as the enzyme is purified. These changes are in the order untreated < DOC-treated < SDS-treated preparations. Since the presence of residual detergent in the membrane would be an important factor in determining membrane fluidity, the preparations were checked after extraction with  $[^{14}\text{C}]$ -sodium deoxycholate and with sodium dodecyl  $[^{35}\text{S}]$ -sulphate followed by the usual washing procedures described under Methods. By using a value of 250,000 for the molecular weight of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  macromolecule (Schwartz *et al.*, 1975) it





was calculated that there were less than 1.5 moles of residual DOC per mole ATPase in these preparations. It is unlikely that this could significantly alter the temperature response of the system. Conversely, the residual SDS was much higher, 150 moles/mole ATPase, and this amount of detergent could significantly influence membrane behaviour. However this interpretation is considerably weakened by the similarity with the results obtained from the liposome study of untreated microsomes which do not contain any detergent at all. While it is possible that the residual SDS may be acting as a significant fluidizer of the membrane lipids in general, this would again be in conflict with the results from the liposome study of the two detergent treated preparations.

The cholesterol content of the total lipid extract of all three types of membranes (untreated, DOC- and SDS-extracted) was also determined by a modified Liebermann-Burchard reaction (Huang *et al.*, 1961). In this study it was found that 16% of the total lipids (dry weight) was cholesterol in the untreated membranes, whereas after DOC and SDS, this value was reduced respectively to 14% and 11%. Whether this decreased cholesterol content coupled to the greater residual detergent that was detected accounts for the loss of correlation in results after SDS treatment cannot be determined from the data.

The results from ESR experiments discussed thus far emphasize three observations:

- a) Increased specific activity after detergent treatment implies a concentration of this integral protein and presumably a concomitant increase of the associated lipids.
- b) Enhancement of specific activity results in an increase in the number of R-binding sites in the bilayer for M 12-NSE.



- c) As determined by the spin probe M 12-NSE the effects of temperature on the spectra of microsomes and liposomes is markedly dissimilar in untreated membranes, and less so after DOC treatment. After treatment with SDS however the correlation between microsomes and liposomes is good. This suggests an increasing homogeneity of the lipids in the order untreated, DOC treated and SDS treated.
- d) It is unlikely that residual detergent is responsible for the altered behaviour of the lipids with changes in temperature.

So the question arises as to the nature of the phospholipid which is retained or concentrated in parallel with the protein macromolecule. It is recalled that brain tissue (the source of our enzyme) which is rich in  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , also contains proportionally higher concentrations of fatty acids with 18-C chains (Chapman, 1975). Therefore it must be considered that the suggested concentration or retention of lipids discussed above may occur by preference for characteristics of the methylene chain rather than by phospholipid head groups alone. This suggestion is also supported by work from other laboratories (Tanaka and Teruya, 1973) and in particular by the recent work of Palatini *et al.*, (1977). In their enzyme reconstitution experiments they showed that dimyristoyl phosphatidyl glycerol with its short chain fatty acids, and not dioleoyl phosphatidyl glycerol with the longer chains, resulted in a non-linear Arrhenius plot of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity.

The apparent lack of correlation between the effect of temperature on the liposomes made from total lipid extracts of the enzyme preparations and their parent micro-



somes clearly suggests an important ordering role for the protein on the lipid.

Thus, in addition to the selective retention of lipid species by the protein the suggestion is also made that the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  macromolecule, which is known to span the membrane, also exerts a powerful co-operative influence on the surrounding assembly of phospholipids.

Yet another possibility remains to be considered; after treatment with SDS the spin probe molecules might become bound to areas of lipid which are unrelated to the enzyme protein, and uninfluenced by the thermal behaviour of their phospholipid neighbours. Such an explanation however, would require migration of the spin labels from the binding sites in the untreated preparations since the latter clearly reflect similar thermal changes in both the hydrolytic activity of the enzyme protein and in the phospholipids of the membranes. Furthermore, a positive correlation between the incidence of R sites and enzyme purity was shown to exist for membrane preparations irrespective of the methods of purification. Thus it seems more likely that in the SDS treated preparations, the lipid "pool" containing the spin probe is adjacent to the enzyme, and whereas the fluidity of this region is reduced by cooling the methylene chains no longer become fully extended within this temperature range.

#### 8. Membrane elasticity.

The preceding discussion has provided the basis for a model in which the co-operative effect between the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  protein and the lipids of the membrane is two fold. First, the protein macro-







molecule exerts an ordering effect on the phospholipids in the bilayer, and secondly, the alterations of the membrane fluidity caused by cooling of the fatty acid chains of the phospholipid in turn impose restraints on the protein macromolecule leading to conformational readjustment.

The data presented in the final section of the chapter on results (section 7) showed a relationship between the changes in membrane fluidity and in the spectrum of M 12-NSE in ox brain microsomes. From the data a number of observations were made and these are reproduced below:

- a) Purification of the enzyme by treating the membranes with SDS, does not alter its thermal sensitivity of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  as determined by the hydrolytic activity.
- b) The changes in molecular motion of the lipid spin probe M 12-NSE qualitatively duplicates the typical temperature phenomenon associated with the hydrolytic activity of this enzyme.
- c) In spin labelled untreated microsomes, the rate of change in mobility of the probe is rapid at high temperatures, and gradual at low temperatures. This result is the opposite of that found in synthetic phospholipid bilayers.
- d) When SDS-treated microsomes are spin labelled, the rate of change in mobility of the probe follows the profile of untreated microsomes, with saltatory cooling only; with slow gradual cooling which presumably allows time for some form of re-equilibration to occur between the probe and its environment these treated microsomes behave like synthetic phospholipid bilayers.



- e) In both the untreated and the detergent treated preparations of the enzyme, the presence of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  protein is essential for this atypical behaviour.

From these observations, the following conclusions can be drawn about  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  containing membranes:

- a) A physical change occurs in the microsomal membrane when it is cooled, which is not normally observed with bilayers made from synthetic phospholipids.
- b) Since microsomes and not liposomes exhibit this 'unique' physical change, an interaction between the protein and its lipid environment must occur.
- c) In SDS-treated membranes, the 'unique' physical change can be eliminated by gradual cooling, and may occur by a protein dependent sequestration of special phospholipids that have become concentrated along with the enzyme.
- d) The conformational changes essential for enzyme activity respond to so-called phase transitions of the membrane lipids, irrespective of the degree of purification.

This information enables an extension of the concept of protein-lipid interaction as it pertains to the temperature sensitivity of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ .

Although well documented, the phase transitions reported for biological membranes are poorly understood. In 1967 Chapman *et al.* showed that the methylene chains of the fatty acids are involved in phase transitions. Above the transition the arrangement of the methylene chains is still uncertain, while below the transition the methylene



## ILLUSTRATIONS OF FIGURES

Fig. 32

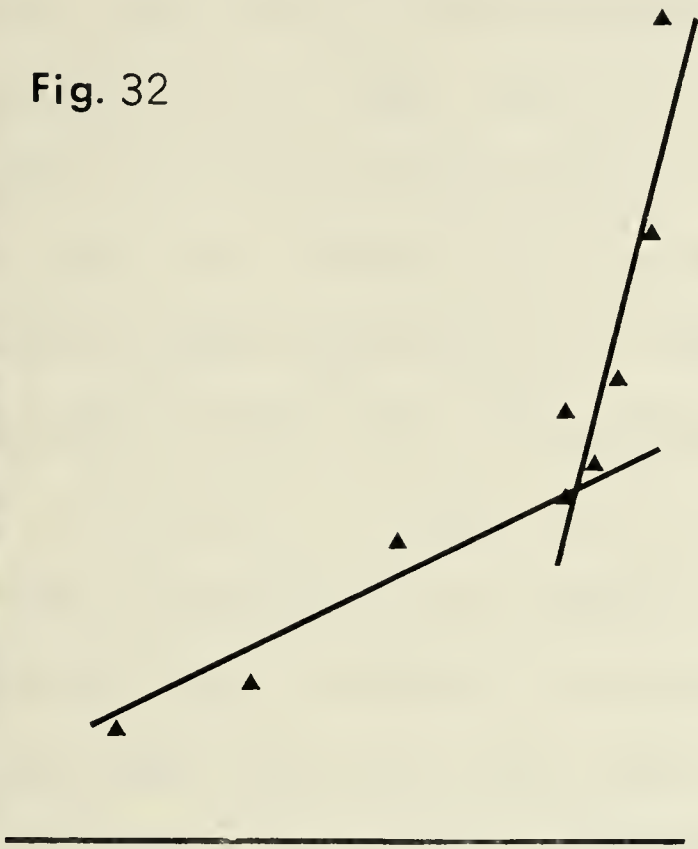


Fig. 33

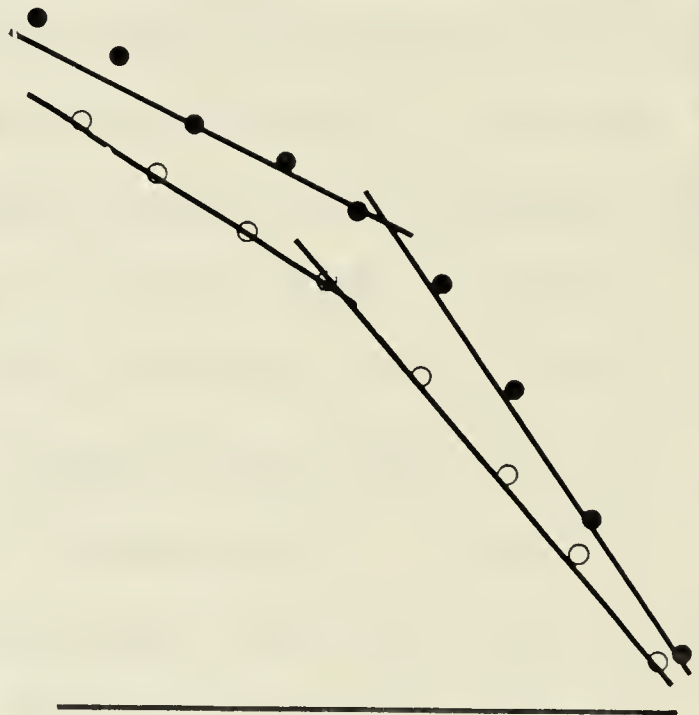


Fig. 34

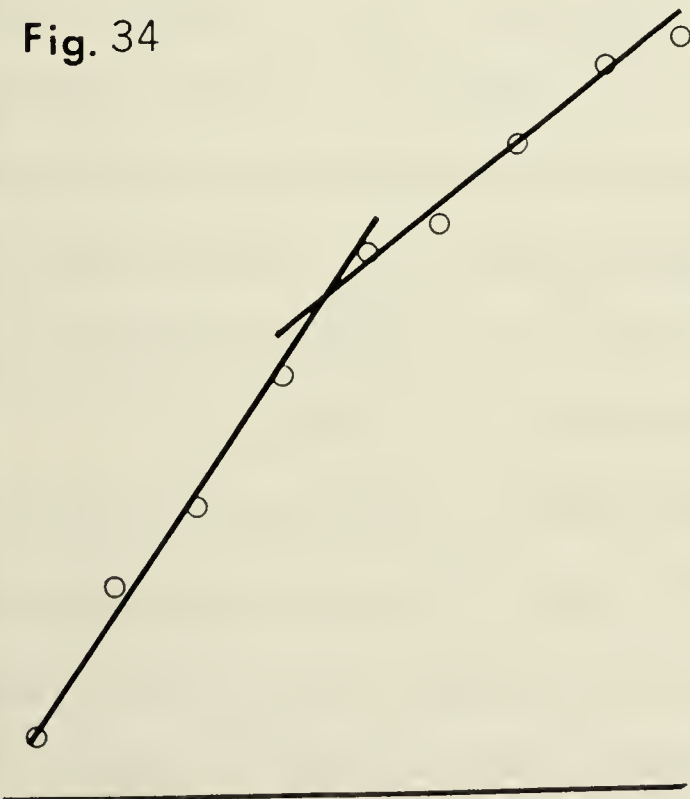
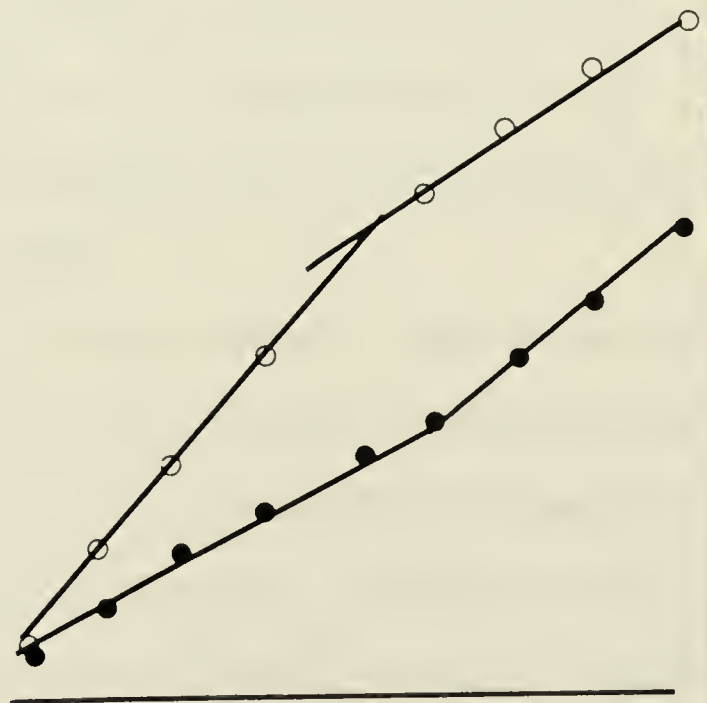


Fig. 35







chains become stiff and lie parallel to each other and perpendicular to the plane of the membrane (Lee, 1977). The stiffening of the methylene chains due to cooling, causes an increase in width of the bilayer (Chapman *et al.*, 1967; Trauble and Haynes, 1971; Pagano *et al.*, 1973). Thus in Fig. 32, the more gradual slope between about 36°C and 21°C reflects a loss of mobility of the methylene chains above  $T_c$ , and the sudden steeper slope below 21°C probably reflects the stiffening of the methylene chains with a concomitant increase in width of the bilayer. It is reasonable to expect that a similar stiffening of the methylene chains accompanied by an increase in membrane width would also occur when biological membranes are cooled (Engleman, 1971). Therefore it seems most likely that when cooled, microsomal membranes reach a critical width at the  $T_c$  (Fig. 34) which leads to an interaction between the  $(Na^+ + K^+)$ -ATPase macromolecule and the phospholipids closely associated with it. This interaction opposes any further increase in this dimension. In the case of the SDS-treated microsomes (Fig. 35, open circles) a similar sequence of events occurs. If however, a slow cooling process is employed (Fig. 35, closed circles), the protein-lipid interaction permits an accumulation of lipid species which by virtue of their chain characteristics [length and(?) unsaturation] do not widen the membrane to the same degree.

In terms of the hydrolytic activity however, there is no difference in the effects of temperature on the untreated or detergent extracted preparations (Fig. 33). Thus, it is a reasonable presumption that the functional aspects of this pump-enzyme are acutely sensitive to changes in membrane width; when a critical membrane width is reached, the enzyme by adopting a new set or series of conformation changes main-



tains continued function, albeit with a decreased thermodynamic efficiency.

These results are compatible with a model in which the membrane fluidity which is essential for enzyme function, can exert its influence in two planes, parallel as well as perpendicular to that of the bilayer. This implies that the membrane behaves like a sheet of rubber with forces acting in the two planes. Weiss (1973) has proposed a "mechanicochemical principle" in his review on the role of lipid in energy transmission (Weiss, 1973). In his concept, Weiss hypothesized that "membrane receptors in lipid induce expansive tendencies at the surface of the membrane which are opposed by a spring-like expansive tendency from the contracted lipid". The concept presented here includes spring-like forces in more than one plane and permits the use of potential energy at any depth in the membrane. Inherent in this hypothesis is the concept that the membrane tends to return to its equilibrium state. It is conceivable then, that the conformational change from  $E_1\text{-P}$  to  $E_2\text{-P}$  by this transmembrane protein macromolecule, the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , could cause an alteration in the balance of the two forces described above. Thus in the case of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  enzyme system, it is likely that the protein moiety incorporates the potential energy of an elastic membrane in the energy transduction process that is necessary for transport.



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## APPENDICES



## APPENDIX I





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## Activation Energy and Phospholipid Requirements of Membrane-Bound Adenosine Triphosphatases<sup>1</sup>

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In order to evaluate the role of lipids in the function of membrane ATPase reactions, the apparent activation energies of membrane-bound ( $\text{Na}^+ + \text{K}^+$ )-ATPase and membrane-bound  $\text{Mg}^{2+}$ -ATPase have been measured under conditions frequently supposed to alter the membrane lipids *in vitro*.

In the case of ( $\text{Na}^+ + \text{K}^+$ )-ATPase, the untreated enzyme was shown to have two different activation energies as shown by an Arrhenius plot comprising two straight lines which intersect at the "critical temperature." Treatment of the preparation with detergents or with phospholipase C causes some alteration in the specific activity of the enzyme but did not significantly alter the activation energies or the critical temperature. After treatment with phospholipase A, however, the Arrhenius plot appeared linear over the entire temperature range studied. Subsequent treatment of phospholipase A-treated preparations with phosphatidylserine restored the control response.

Conversely, untreated preparations of  $\text{Mg}^{2+}$ -ATPase give an Arrhenius plot which is neither linear nor composed of two intersecting straight lines. This plot, which we regard as curvilinear, does not permit a unique value of the activation energy to be determined. The shape of this plot is unaltered by detergent or by treatment with phospholipase C. In contrast to ( $\text{Na}^+ + \text{K}^+$ )-ATPase, it is also unaffected by treatment with phospholipase A or phospholipase A followed by phosphatidylserine.

We conclude that although ( $\text{Na}^+ + \text{K}^+$ )-ATPase and  $\text{Mg}^{2+}$ -ATPase are frequently closely associated in many membranes, their functions involve the presence of different membrane lipids.

The recent studies of Raison and Lyons (1, 2) have shown that the properties of some membrane-bound enzymes are greatly influenced by the physical state of the membrane lipids. In particular, these workers have demonstrated the close relationship which exists between enzyme activity, the apparent activation energy of the system, and the motional freedom of the membrane lipids (1, 2).

It therefore seems likely that the sudden "breaks" or sharp departures from linearity that have been observed in the Arrhenius plots of membrane-bound ( $\text{Na}^+ + \text{K}^+$ )-

ATPase (3-6) prepared from a variety of sources might both result from, and be influenced by, the type and state of the phospholipids in the membranes. This enzyme system would seem to be well suited to the study of this phenomenon as it has long been known that its activity can be reduced by a variety of treatments which remove lipids or their components from membranes (7-9), and that activity can frequently be restored by replacement of either phospholipids (10-14) or cholesterol (15, 16) to the system.

This paper describes the effects of a variety of lipid extraction procedures upon the apparent activation energy of ( $\text{Na}^+ + \text{K}^+$ )-ATPase both above and below the critical

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temperature for the system, and the effect of phosphatidylserine replacement following treatment of the membrane preparations with phospholipases. The effect of these treatments on the activation energy of  $\text{Mg}^{2+}$ -ATPase, which was always associated with these preparations, is also described.

#### MATERIALS AND METHODS

"Heavy" microsomal membrane preparations rich in ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase but also containing ouabain-insensitive  $\text{Mg}^{2+}$ -ATPase activity were obtained from the renal cortex of mature New Zealand white rabbits by the procedure first described by Charnock and Post (17). The membrane enzyme preparations were suspended and stored at  $4^\circ\text{C}$  in a sucrose-buffer mixture of 0.275 M sucrose and 0.2 mM EDTA adjusted to pH 7.6 by the addition of 1 M Tris-base. The protein content of these enzyme suspensions was determined by the method of Lowry *et al.* (18) and was maintained between 1 and 3 mg protein/ml.

Both the ouabain-sensitive and ouabain-insensitive ATPase activity of the freshly prepared microsomal membrane fractions was obtained by the assay procedure of Charnock and Post (17) both prior to and after other experimental procedures described below. The assay conditions were adjusted so that the reaction velocity obtained was constant throughout the incubation periods employed, and closely approaches the  $V_{\max}(t^0)$  for both ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase and  $\text{Mg}^{2+}$ -ATPase. Excess substrate ( $\text{Mg}\cdot\text{ATP}$ ) was always present, and the concentration of ADP generated by the reaction was never allowed to exceed 20% of that of the initial concentration of ATP. The reaction velocities so obtained were converted to rate constant ( $\mu\text{moles P}_i$  liberated/mg protein/hr) before calculation of the Arrhenius graphs of temperature dependence of these enzyme systems.

*Extraction with deoxycholate.* Two volumes of freshly prepared membrane suspension (3 mg protein/ml) were mixed with 1 vol of 0.2% Tris-deoxycholate (pH 7.2) and allowed to stand for 16 hr at  $4^\circ\text{C}$ . The extracted membranes were recovered by centrifugation at 40,000g for 30 min and resuspended in the sucrose-buffer described above.

*Extraction with Nonidet P40.* One volume of freshly prepared membrane suspension was mixed with 4 vol of 1% Nonidet P40 nonionic detergent (kindly supplied by Dr. J. K. Raison) and allowed to stand for 30 min at  $4^\circ\text{C}$ . The extracted membranes were recovered and resuspended as above.

*Phospholipase A.* When necessary, the membrane suspension was diluted with sucrose buffer to 1 mg protein/ml and was incubated with 2.67  $\mu\text{g/ml}$  of bee venom phospholipase A (Sigma Chemical Co.) in a medium adapted from that described

by Imai and Sato (19). The final concentration of reagents in the incubation solution was: bovine serum albumin, 7.35 mg/ml; Tris base, 14.7 mM; 2-mercaptoethanol, 33.7 mM; EDTA, 0.035 mM;  $\text{CaCl}_2$ , 5.6 mM; sucrose, 0.55 M; adjusted to pH 7.4 by the addition of 1 M Tris base. After 5 min incubation at  $37^\circ\text{C}$  to insure temperature equilibration of all components of the system, the reaction was started by the addition of phospholipase A. After a further 12 min incubation at  $37^\circ\text{C}$  the reaction was stopped by the addition of 2 vol of 57 mM EDTA-321 mM Tris (adjusted to pH 7.4 by addition of 1 M HCl) to 3 vol of reaction mixture and immediately diluted to 200 ml with ice-cold distilled water. The particulate membrane fraction was then recovered by centrifuging at 77,500g for 3 hr and resuspending in the sucrose buffer solution employed before to again yield a protein content of 1 mg/ml. Some of these phospholipase A-treated preparations were then incubated for 10 min at  $37^\circ\text{C}$  with an equal vol of phosphatidylserine (3 mg/ml) immediately before the determination of temperature dependence described below.

*Phospholipase C.* The membrane suspension was diluted to a protein content of 0.8 mg/ml and incubated with 0.2 mg phospholipase C (prepared from *Clostridium welchii* by the Sigma Chemical Co.) in a mixture containing a final concentration of imidazole, 12.5 mM;  $\text{CaCl}_2$ , 3.7 mM; NaCl, 64.0 mM; KCl, 16.0 mM; and  $\text{MgSO}_4$ , 4.8 mM (adjusted to pH 7.1 with 1 M HCl) according to the method of Smith and Kemp (20). After bringing to constant temperature the reaction was started by the addition of phospholipase C and incubated for 1 hr at  $37^\circ\text{C}$ . The reaction was stopped by cooling to  $4^\circ\text{C}$ , and the particulate membrane fraction recovered by centrifugation at 40,000g for 30 min. The pellet was resuspended in sucrose buffer as described above.

*Activation energy.* The effect of temperature on ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase activity and ouabain-insensitive  $\text{Mg}^{2+}$ -ATPase activity of both the untreated and treated membrane preparations was determined by the procedure we have described previously (4) and the data so obtained used to construct Arrhenius graphs from  $5^\circ$  to  $37^\circ\text{C}$ . These graphs were again analyzed with the assistance of the APL360 computer program developed in this laboratory (4) to yield values for the apparent activation energies and critical temperatures of both ATPase reactions, and to provide the statistical confidence limits for the number of lines which can be fitted to the plots.

#### RESULTS

*Ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase.* In agreement with our earlier findings (17) the specific activity of ouabain-sensitive ( $\text{Na}^+ +$





K<sup>+</sup>)-ATPase was increased about twofold after 16-hr extraction with a low concentration (less than 0.1%) of deoxycholate (DOC). A similar but quantitatively greater effect was obtained after 30-min extraction with 0.2% Nonidet P40 when enzyme activity was enhanced about fivefold. The incubation conditions for treatment with either phospholipase A or phospholipase C were adjusted after preliminary experiments, so that any changes in enzyme specific activity were relatively small when compared to the effects obtained with Nonidet P40. These results are shown in Table I.

The apparent activation energies of detergent-treated enzyme preparations were calculated from the Arrhenius graphs shown in Fig. 1 and were not significantly different from those of the untreated control preparations. The calculated mean values for the apparent activation energies for these enzyme preparations above ( $E_{aI}$ ) and below ( $E_{aII}$ ) the critical temperature ( $T_c^\circ$ ) are given in Table II.

Although brief incubation of the membranes with phospholipase A was without significant effect upon the specific activity of the enzyme preparations at 37°C, there was a marked effect upon the temperature dependence of the reaction velocity over the whole experimental range. This is shown by the Arrhenius graph given in Fig. 2 where

TABLE I  
THE EFFECT OF VARIOUS TREATMENTS ON THE ACTIVITY OF MEMBRANE ATPASE

Conditions <sup>a</sup>	% Ouabain-insensitive Mg <sup>2+</sup> -ATPase	% Ouabain-sensitive (Na <sup>+</sup> + K <sup>+</sup> )-ATPase
Untreated control	100	100
Deoxycholate	126	212 <sup>b</sup>
Nonidet P40	186 <sup>b</sup>	520 <sup>b</sup>
Phosphatidylserine	88	150
Phospholipase A	102	130
Phospholipase A plus phosphatidylserine	96	82
Phospholipase C	113	158 <sup>b</sup>

<sup>a</sup> Details of treatment are given under *Materials and Methods*. Values given are the means of at least six separate experiments.

<sup>b</sup> Value significantly different from untreated control ( $P < 0.05$ ).

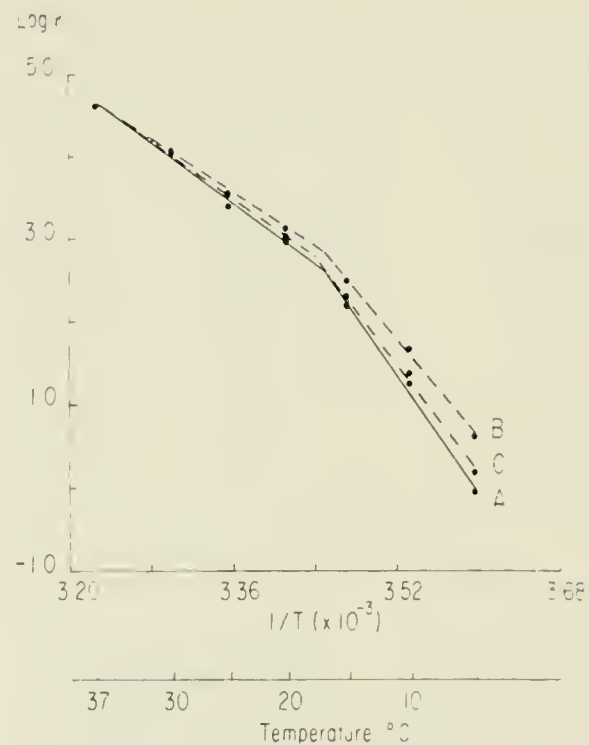


FIG. 1. Arrhenius graphs of inorganic phosphate liberation from ATP by ouabain-sensitive (Na<sup>+</sup> + K<sup>+</sup>)-ATPase before and after detergent treatment. The data points were obtained by calculation of the difference in activity of the enzyme preparations assayed with 80 mM Na<sup>+</sup>, 20 mM K<sup>+</sup>, and 4 mM Mg·ATP  $\pm$  1 mM ouabain. Plot A is the control before detergent treatment; Plot B is after extraction with DOC and Plot C is after extraction with Nonidet P40. The values for the apparent activation energies which were derived from these plots are given in Table II.

the sharp "breaks" in the plot which previously characterized these preparations are no longer apparent. Computer-assisted analysis of the data (4) indicated that a single straight line can now be fitted to the data points with a high degree of statistical confidence. This linear relationship yields a unique value of about 24 kcal/mole for the apparent activation energy of treated (Na<sup>+</sup> + K<sup>+</sup>)-ATPase determined between 5–37°C (Table II).

Reconstitution experiments with phosphatidylserine after phospholipase A treatment resulted in the reappearance of the "break" in these plots and returned the temperature dependence of the enzyme to that indistinguishable from the untreated controls. Addition of phosphatidylserine alone to untreated enzyme preparations was without significant effect upon either enzyme specific activity or temperature dependence; the values for  $E_{aI}$  and  $E_{aII}$  are given in Table II.





TABLE II  
APPARENT ACTIVATION ENERGY OF  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  AFTER VARIOUS TREATMENTS

Conditions	No. of experiments	$E_{aI}$ above $T_c^\circ$ <sup>a</sup>	$E_{aII}$ below $T_c^\circ$ <sup>a</sup>	$T_c^\circ$
Untreated control	6	$19.1 \pm 1.3$	$43.1 \pm 3.9$	$16.5 \pm 1.5$
Deoxycholate	6	$17.8 \pm 1.7$	$36.3 \pm 4.2$	$18.5 \pm 2.3$
Nonidet P40	13	$16.4 \pm 0.5$	$37.2 \pm 5.1$	$19.5 \pm 1.8$
Phospholipase A	7	$23.9 \pm 1.5^b$	$24.0 \pm 3.9^b$	—
Phospholipase A and phosphatidylserine	5	$20.2 \pm 1.1$	$43.6 \pm 7.2$	$18.7 \pm 0.5$
Phosphatidylserine	3	$19.5 \pm 1.2$	$39.7 \pm 1.7$	$20.4 \pm 1.2$
Phospholipase C	5	$14.1 \pm 0.9$	$25.9 \pm 1.4^b$	$16.4 \pm 0.9$

<sup>a</sup>  $E_a$  given as kcal/mole  $\pm$  standard error.

<sup>b</sup> Values for  $E_{aI}$  and  $E_{aII}$  are significantly different ( $P < 0.05$ ) from untreated controls.

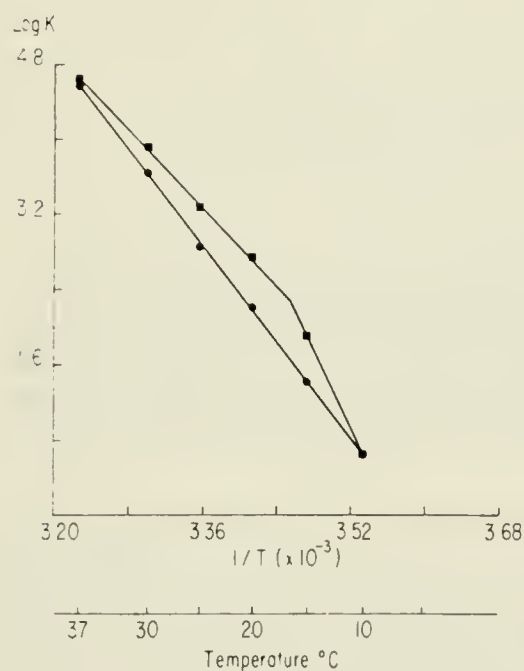


FIG. 2. Arrhenius graphs of inorganic phosphate liberation from ATP by ouabain-sensitive  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  after treatment with phospholipase A. The lower plot (●) shows the effect after short-term treatment with phospholipase A where the linear plot no longer displays a critical temperature. The upper plot (■) shows the effect of phosphatidylserine addition after treatment with phospholipase A and again displays the nonlinearity of the response similar to that of the untreated control shown in Fig. 1. The values for the apparent activation energies which were derived from these plots are given in Table II.

Treatment with phospholipase C gave results qualitatively similar to those obtained for the controls, but resulted in a decrease in the apparent activation energy of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  below  $T_c^\circ$  as was also seen after treatment with phospholipase A (Table II).

Subsequent addition of phosphatidylserine was without effect.

*Ouabain-insensitive  $\text{Mg}^{2+}\text{-ATPase}$ .* The specific activity of ouabain-insensitive  $\text{Mg}^{2+}\text{-ATPase}$  was significantly increased by treatment with Nonidet P40, but was not markedly changed by treatment with DOC, phosphatidylserine, phospholipase A, or phospholipase C. The activities observed before and after treatment are given in Table I. The effect of temperature on the reaction velocity of  $\text{Mg}^{2+}\text{-ATPase}$  of untreated microsomal membrane preparations was examined in six separate experiments. The mean reaction velocities at temperatures from 5 to 37°C were obtained and used to construct the Arrhenius plot shown in Fig. 3a. A computer-assisted analysis of this data suggested that the plot was not linear over this range, nor could it be fitted by two intersecting straight lines with a high degree of confidence. Seemingly, this data is most reasonably regarded as being curvilinear between 5–37°C, a conclusion which precludes the assignment of a meaningful unique value for the activation energy of this process over this range. However, the data will allow an estimate of the range of values which can be obtained. For example, the values estimated from the control curve shown in Fig. 3a extend from 12.0 kcal/mole at higher temperatures to 25.5 kcal/mole at the lower temperatures. Incubation with phosphatidylserine alone did not significantly alter this observation, again yielding a curvilinear plot shown in Fig. 3b, with a similar estimated



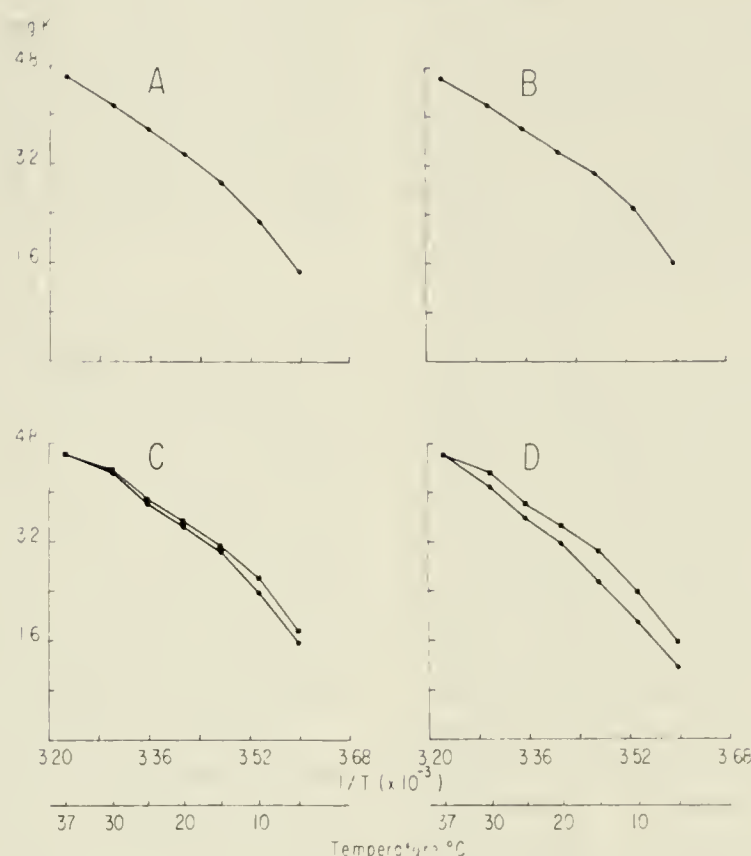


FIG. 3. Arrhenius graphs of inorganic phosphate liberation from ATP by ouabain-insensitive  $\text{Mg}^{2+}$ -ATPase after various treatments. (A) untreated control; (B) after addition of phosphatidylserine alone; (C) lower curve (●) after Nonidet P40; upper curve (■) after DOC; (D) lower curve (●) after phospholipase A; upper curve (■) after phospholipase A followed by addition of phosphatidylserine. The values for the range of apparent activation energies which were derived from these plots are given in the text.

range of values for activation energy (12.2–23.6 kcal/mole).

Extraction with deoxycholate was also without effect on the temperature dependence of  $\text{Mg}^{2+}$ -ATPase, as the Arrhenius plot obtained after treatment with this agent also appeared to be curvilinear, again yielding values estimated between 11.3–25.1 kcal/mole. Similarly the data obtained after Nonidet P40 extraction did not show any effect of this agent upon the temperature dependence of ouabain-insensitive ATPase activity; the Arrhenius plots after treatment with DOC and Nonidet P40 being virtually identical (Fig. 3c).

However, unlike the findings with ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase, there was also no significant change in the temperature dependence of ouabain-insensitive  $\text{Mg}^{2+}$ -ATPase after treatment with phospholipase A or after treatment with phospholipase A followed by reincubation with phosphatidylserine (Fig. 3d). That is, the Arrhe-

nus plots obtained after treatment with these agents could neither be fitted by a single straight line nor by two intersecting straight lines with any acceptable degree of statistical confidence. Treatment with phospholipase C was also without effect upon the temperature dependence of  $\text{Mg}^{2+}$ -ATPase and is not shown in Fig. 3.

Thus, all of these experiments failed to yield a meaningful unique value for the apparent activation energy of the reaction, but gave a maximum range of estimated values from 11.3 kcal/mole at the high temperatures to 29.4 kcal/mole at the lower temperatures.

#### DISCUSSION

In previous reports from this laboratory (3, 4) we have shown that the temperature dependence of ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase is nonlinear between 5–37°C; resulting in two widely different apparent activation energies above and below a criti-





cal temperature of about 18°C. This finding is confirmed in the present study. We have also shown that these apparent activation energies are unchanged by alterations in the total cation concentration of the incubation medium, or by different periods of storage which produce variable decreases in enzyme specific activity (4).

Our present findings, in which detergent treatment results in large increases in enzyme specific activity without change in apparent activation energies of this enzyme, reinforce our previous conclusion that this thermodynamic parameter is independent of the enzyme specific activity and thus does not reflect the number of *active centers* which are operational. Rather it is a measure of the functional ability of individual sites and, as such, can be influenced by molecular changes within the membrane matrix.

After brief incubation with phospholipase A which was adjusted to maintain the specific activity of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  at or near the control level, there was a marked change in the temperature dependence of this enzyme which was now linear between 5° and 37°C, thus giving a unique value for the apparent activation energy of this membrane bound enzyme of approximately 24 kcal/mole.

In addition, the effect of treatment with phospholipase A could be reversed by reincubating the lipase-treated membranes with phosphatidylserine. Similar findings have recently been reported by several other workers (6, 21) who have independently confirmed the reactivating action of this phospholipid upon lipase-treated membrane preparations. However, the selectivity of phosphatidylserine for this effect has not been demonstrated as partial reactivation of membrane ATPase has also been reported with phosphatidylethanolamine (11) and lysolecithin (12). More recently the studies of Noguchi and Freed (15) and Jarnefelt (16) have shown that cholesterol may have a similar action following organic solvent extraction of the membranes.

In this regard it is of interest that treatment of the membranes with phospholipase C did not result in the conversion of the temperature dependence of ouabain-sensi-

tive  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  to a linear relationship, suggesting that a selective cleavage of the phospholipids involved in this phenomenon is an important determinant in this effect. It is not surprising, therefore, that addition of phosphatidylserine after treatment with phospholipase C was without effect upon the apparent activation energy of the enzyme.

Conversely, neither treatment with detergents nor lipases resulted in any detectable change in the temperature dependence of the ouabain-insensitive  $\text{Mg}^{2+}\text{-ATPase}$  associated with these membrane preparations. This latter enzyme function could not be described as being either linear or be adequately fitted by two linear subsections separated by a critical temperature. Seemingly our conclusion from the examination of the data from separate experimental situations is valid, and that when all the data from more than 40 experiments is pooled, the resultant Arrhenius plot of ouabain-insensitive  $\text{Mg}^{2+}\text{-ATPase}$  must be regarded as a curve; that is, the temperature response of this enzyme is demonstrably different from that obtained with ouabain-sensitive  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . This finding suggests that the phospholipids closely associated with the *active centers* of ouabain-sensitive *transport ATPase* are either distinct from those associated with the physiologically less well-defined ouabain-insensitive  $\text{Mg}^{2+}\text{-ATPase}$  enzyme system, or, somewhat less probably, there are no phospholipids associated with  $\text{Mg}^{2+}\text{-ATPase}$  activity.

However, whichever of these suggestions is correct, it seems reasonable to conclude that the now well-established requirement of ouabain-sensitive  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  for phospholipid (8, 10-12) as well as the determining role of phospholipid in the temperature dependence of this system, must now be regarded as characteristics which distinguish this "transport enzyme" from the  $\text{Mg}^{2+}$ -dependent but ouabain-insensitive *basal ATPase* with which it is closely associated within the membrane matrix.

It is also reasonable to conclude that the influence of phospholipids upon the activation energy of ouabain-sensitive *transport ATPase* must have profound implications





for active sodium transport mechanisms in hibernating mammals and other temperature dependent species (22-24).

#### ACKNOWLEDGMENT

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## APPENDIX II



## Temperature-Activity Relationships of Cation Activation and Ouabain Inhibition of ( $\text{Na}^+ + \text{K}^+$ )-ATPase

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The nonlinear temperature-activity relationship of membrane preparations of ( $\text{Na}^+ + \text{K}^+$ )-ATPase gives rise to discontinuities in Arrhenius plots of this enzyme. The different apparent energies of activation of ( $\text{Na}^+ + \text{K}^+$ )-ATPase which are observed above and below the critical temperature of the system have been considered to result from different conformational forms of the enzyme protein. Because both activation of ( $\text{Na}^+ + \text{K}^+$ )-ATPase by cations, and its specific inhibition by cardiac glycosides may be influenced by the conformational form of the enzyme protein, we have reexamined the effect of temperature upon the activation energy of the system under the different experimental conditions of cation activation and ouabain inhibition.

Our results indicate that the activation of ( $\text{Na}^+ + \text{K}^+$ )-ATPase by cations, is less influenced by change in temperature than is inhibition of the enzyme by ouabain. In addition, mild lipolysis by phospholipase-A had a marked effect upon the ouabain-dependent response of the enzyme to temperature, but not upon the cation-dependent response. The effect of phospholipase-A can be overcome by reincubation of the treated preparation with phosphatidyl serine.

We conclude that the ouabain-dependent temperature effects of ( $\text{Na}^+ + \text{K}^+$ )-ATPase are more dependent upon the integrity and nature of the membrane lipids than are the cation-dependent responses. It is possible that phosphatidyl serine plays a unique role in this regard.

It has been known for many years that the activity of membrane-bound ( $\text{Na}^+ + \text{K}^+$ )-ATPase is dependent upon the amount and composition of the membrane lipids (1-4). Procedures such as treatment with phospholipases, or extraction with detergents or lipid solvents, frequently lead to either partial or total inactivation of the enzyme system (5-9). Very recently the work of several investigators has demonstrated that the temperature-activity relationship of this enzyme is also dependent upon the nature and physical state of the membrane lipids (10-16). Phosphatidyl serine has been shown to overcome the effect of partial removal of a lipid moiety by either phospholipase-A (11, 12) or vigorous treatment with deoxycholate (14, 17, 18).

Discontinuities in Arrhenius plots of

( $\text{Na}^+ + \text{K}^+$ )-ATPase activity are also considered to arise from different conformational forms of the enzyme protein above and below the critical temperature of the system (19, 20). However, it is now clear that the membrane lipids, and particularly phosphatidyl serine, play a major role in determining the conformational form and activation energy of ( $\text{Na}^+ + \text{K}^+$ )-ATPase (10-12). Because both the activation of ( $\text{Na}^+ + \text{K}^+$ )-ATPase by cations, and its specific inhibition by ouabain, are thought to be influenced by the conformational form of the enzyme protein (20) we have re-examined the effects of cation activation and ouabain inhibition on ( $\text{Na}^+ + \text{K}^+$ )-ATPase activity as a function of temperature, in order to examine this hypothesis further.





## MATERIALS AND METHODS

The methods for the preparation of ( $\text{Na}^+ + \text{K}^+$ )-ATPase from rabbit kidney cortex (21) and the treatments with lipases or detergents (12) have been described in detail in previous publications. Treatment of the enzyme preparation with 2 M NaI was carried out according to the procedure described by Nakao *et al.* (22). The assay of ( $\text{Na}^+ + \text{K}^+$ )-ATPase was again carried out by the general procedure we have used before (12), but additional reaction tubes were included so that activation of the enzyme by 80 mM  $\text{Na}^+$  plus 20 mM  $\text{K}^+$  could be determined separately from the inhibition of the enzyme obtained with  $10^{-4}$  M ouabain. Blanks for the possible effect of ouabain upon  $\text{Mg}^{2+}$ -dependent basal ATPase (12) were always included in the assay system, and the values shown in the tables and text of this paper were corrected for any hydrolysis of ATP by this source. Arrhenius plots of the temperature-activity relationships of cation activation and ouabain inhibition of ( $\text{Na}^+ + \text{K}^+$ )-ATPase were obtained by the procedures we have described previously (12). The values for activation energy ( $E_{a1}$  and  $E_{a11}$ ) above and below the critical temperature ( $T^{\circ}\text{c}$ ) were determined with the aid of the computer program we have developed in this laboratory (10). The mean values for  $E_{a1}$ ,  $E_{a11}$  and  $T^{\circ}\text{c} \pm$  their standard errors, were compared by the Student's *t* test.

## RESULTS

The general characteristics of the enzyme preparations from rabbit kidney cortex which were used in this study are shown in Table I. At 37°C, about 80% of the total ATPase activity of the heavy microsomal membrane preparation is due to ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase.

TABLE I

CATION ACTIVATION AND OUABAIN INHIBITION OF RABBIT KIDNEY PREPARATIONS OF ( $\text{Na}^+ + \text{K}^+$ )-ATPase<sup>a</sup>

Assay conditions	% Activity <sup>b</sup>
Enzyme omitted	<0.5
$\text{Mg}^{2+}$	18
$\text{Mg}^{2+} + \text{ouabain}$	15
$\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$	100
$\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+ + \text{ouabain}$	19

<sup>a</sup> Enzyme preparation not treated with detergent or lipase, but stored at  $-10^{\circ}\text{C}$  for 5 days before assay.

<sup>b</sup> All assays determined at 37°C for 15 min. Values given are the means of at least five preparations, with determinations performed in duplicate. The mean specific activity of the enzyme is  $23.7 \pm 1.9$   $\mu\text{mol Pi/mg protein/h}$ .

*Untreated ( $\text{Na}^+ + \text{K}^+$ )-ATPase*

The effect of temperature upon the apparent activation energy of *untreated* preparations of rabbit kidney ( $\text{Na}^+ + \text{K}^+$ )-ATPase is shown as an Arrhenius plot in Fig. 1. The characteristic discontinuity in this plot is again apparent, whether the enzyme activity is determined by activation by cations (80 mM  $\text{Na}^+ + 20$  mM  $\text{K}^+$ ) or by inhibition by cardiac glycoside ( $10^{-4}$  M ouabain), thus confirming our earlier findings (10, 12) and those of other workers (11, 13). However, it should be noted that the apparent activation energies of the enzyme which can be derived from these plots are not identical under the two experimental situations being compared here. Although the divergence in values of  $E_{a1}$  above the critical temperature are much smaller than the divergence in values of  $E_{a11}$  below the critical temperature, the values for both  $E_{a1}$  and  $E_{a11}$  by cation activation are significantly different from those obtained by ouabain inactivation ( $p < .05$ ). Interestingly, the points of inflection in the Arrhenius plots, that is the

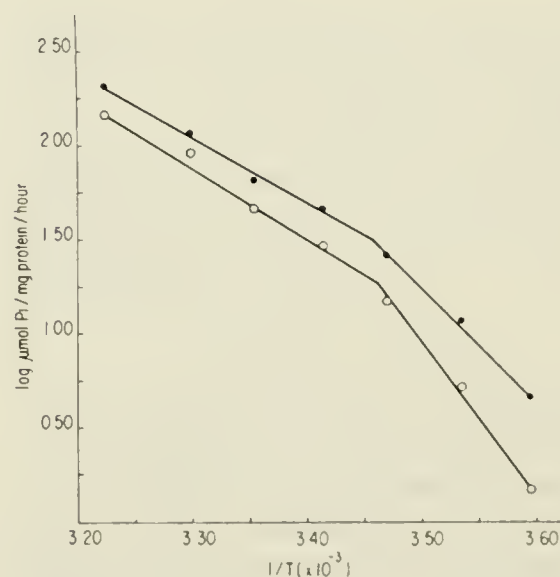


FIG. 1. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●—●) and ouabain inhibition (○—○) of untreated membrane preparations of ( $\text{Na}^+ + \text{K}^+$ )-ATPase. The mean critical temperature ( $T^{\circ}\text{c}$ ) for four experiments is  $16.7 \pm 1.6^{\circ}\text{C}$  and is identical for all experimental conditions. The computed values for  $E_{a1}$  above  $T^{\circ}\text{c}$  are  $15.4 \pm 0.7$  and  $19.1 \pm 1.3$  kcal/mol for activation by cations and inhibition by ouabain, respectively. The values for  $E_{a11}$  below  $T^{\circ}\text{c}$  are  $27.5 \pm 1.9$  and  $43.1 \pm 3.9$  kcal/mol, respectively.



computed critical temperatures ( $T^{\circ}\text{C}$ ) occur at about  $17^{\circ}\text{C}$ , and are not significantly different under these two experimental conditions.

### Detergent Treatment

Previous studies from this laboratory have shown that *mild* treatment with the detergents deoxycholate or Nonidet P40 resulted in significantly increased hydrolytic activity of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , but no change in the temperature-activity relationship or the apparent activation energy of the system (12). In this study the Arrhenius plots that were obtained by both cation activation and ouabain inhibition of the system after partial removal of membrane lipids by deoxycholate, or 0.2% Nonidet P40 are shown in Figs. 2 and 3, respectively. The results obtained are very similar to those reported previously (12); again there is a divergence in the apparent activation energies of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  when determined by either cation

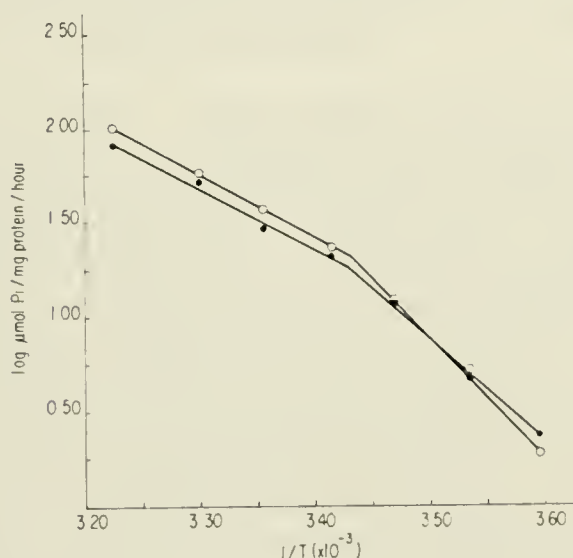


FIG. 2. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●—●) and ouabain inhibition (○—○) of membrane preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  after treatment with 0.1% deoxycholate.

The mean critical temperature ( $T^{\circ}\text{C}$ ) for four experiments is  $18.7 \pm 1.4^{\circ}\text{C}$ , and is identical for all experimental conditions.

The computed values for  $E_{a1}$  above  $T^{\circ}\text{C}$  are  $17.5 \pm 2.1$  and  $17.8 \pm 1.7$  kcal/mol for activation by cations and inhibition by ouabain, respectively.

The values for  $E_{a11}$  below  $T^{\circ}\text{C}$  are  $30.4 \pm 5.9$  and  $36.3 \pm 4.2$  kcal/mol, respectively.

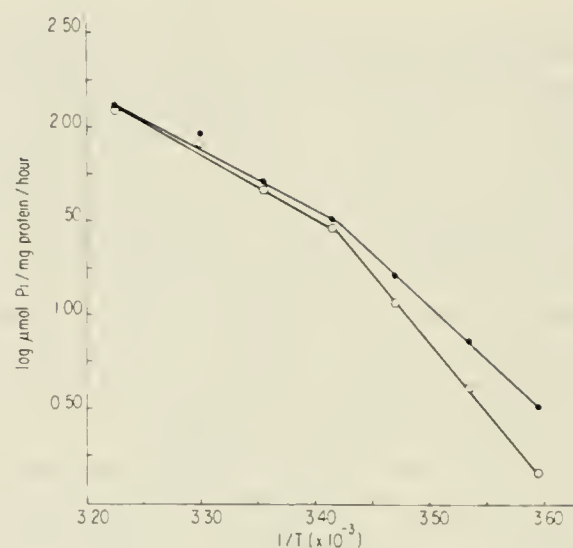


FIG. 3. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●—●) and ouabain inhibition (○—○) of membrane preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  after treatment with 0.2% Nonidet P40.

The mean critical temperature ( $T^{\circ}\text{C}$ ) for four experiments is  $19.5 \pm 1.2^{\circ}\text{C}$  and is identical for all experimental conditions.

The computed values for  $E_{a1}$  above  $T^{\circ}\text{C}$  are  $15.6 \pm 0.6$  and  $16.4 \pm 0.5$  kcal/mol for activation by cations and inhibition by ouabain, respectively.

The values for  $E_{a11}$  below  $T^{\circ}\text{C}$  are  $25.6 \pm 1.0$  and  $37.2 \pm 5.1$  kcal/mol, respectively.

activation or by ouabain inhibition. These results are also similar to those shown in Fig. 1 for untreated membrane preparations. The divergence in apparent activation energies is always greater in the temperature range below the critical temperature which, in this group of experiments is about  $19^{\circ}\text{C}$ .

### Treatment with NaI

After treatment with 2 M NaI (22), the temperature-activity relationship of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  is qualitatively similar to that of the untreated controls when enzyme activity has been determined by inhibition with  $10^{-4}$  M ouabain. There is a sharp discontinuity in the Arrhenius plot at a mean temperature of  $14.3 \pm 5.9^{\circ}\text{C}$ . The apparent energy of activation above this critical temperature is  $20.6 \pm 2.1$  kcal/mol and  $56.2 \pm 24.2$  kcal/mol below  $T^{\circ}\text{C}$  (Fig. 4). These values are not significantly different from those previously obtained under these experimental conditions (12).





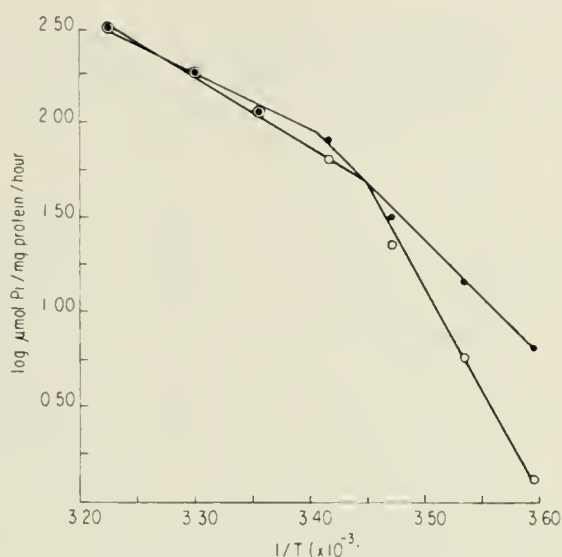


FIG. 4. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●—●) and ouabain inhibition (○—○) of membrane preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  after treatment with 2 M NaI. The mean critical temperature ( $T^\circ\text{c}$ ) for three experiments is  $20.6 \pm 4.1^\circ\text{C}$  for activation by cations, and is  $14.3 \pm 5.9^\circ\text{C}$  when the enzyme activity is determined by inhibition with ouabain.

The computed values for  $Ea_1$  above  $T^\circ\text{c}$  are  $15.4 \pm 1.1$  and  $20.6 \pm 2.1$  kcal/mol for activation by cations and inhibition by ouabain, respectively.

The values for  $Ea_{11}$  below  $T^\circ\text{c}$  are  $27.0 \pm 1.0$  and  $56.2 \pm 24.2$  kcal/mol, respectively.

Conversely, when enzyme activity is determined by activation with cations, some change is apparent in the observed temperature-activity relationship. Although a discontinuity in the Arrhenius plot is still apparent, this now occurs at a significantly higher temperature of  $20.6 \pm 4.1^\circ\text{C}$  than in the untreated controls ( $p < .05$ ). The apparent activation energy above the critical temperature is now  $15.4 \pm 1.1$  kcal/mol, and that below  $T^\circ\text{c}$  is  $27.0 \pm 1.0$  kcal/mol. All these values are significantly different from those of the untreated control (Fig. 1).

#### Lipase Treatment

Although it is well-known that prolonged digestion of biological membranes with phospholipases will completely inactivate membrane preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  (1, 13, 23, 24, 25), we have shown previously that under very mild conditions, treatment with phospholipase-C does not significantly change the ATPase activity of these preparations compared to

appropriate controls (12). Although phospholipase-C is known to interact with many acidic phospholipids, we have again demonstrated in this study that mild treatment with this lipase did not change the temperature-activity relationship of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , when determined by either cation activation or by ouabain inhibition (Fig. 5). Under both conditions,  $Ea_{11} > Ea_1$ . Again the activation energy of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  when activated by cations was significantly less than the value obtained when the enzyme was inhibited by  $10^{-4}$  M ouabain ( $p < .05$ ). Like the effect seen after treatment with NaI, the critical temperature now found in the presence of cations was higher in these preparations than that seen with untreated controls.

Conversely, mild treatment of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  with phospholipase-A produced a marked effect on the temperature-activity relationship of the enzyme when determined by ouabain inhibition. There was a complete loss of the

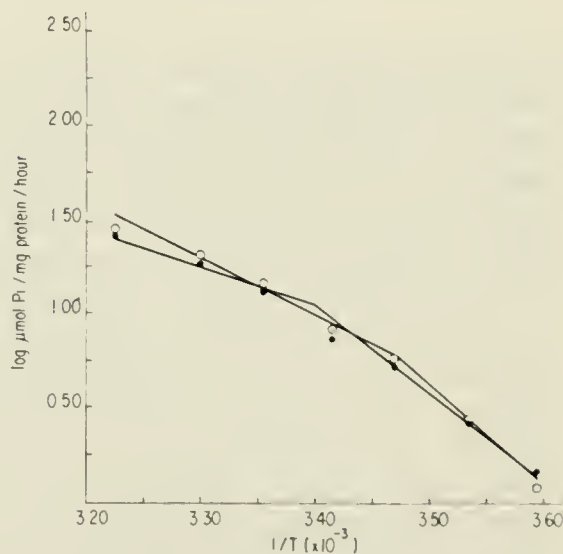


FIG. 5. Arrhenius plots of inorganic phosphate liberation of ATP by cation activation (●—●) and ouabain inhibition (○—○) of membrane preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  after treatment with phospholipase-C.

The mean critical temperature ( $T^\circ\text{c}$ ) for four experiments with cation activation is  $21.2 \pm 3.4^\circ\text{C}$ , and  $16.4 \pm 0.9^\circ\text{C}$  for inhibition by ouabain.

The computed values for  $Ea_1$  above  $T^\circ\text{c}$  are  $11.4 \pm 0.4$  and  $14.1 \pm 0.9$  kcal/mol for activation by cations and inhibition by ouabain, respectively. The values for  $Ea_{11}$  below  $T^\circ\text{c}$  are  $20.0 \pm 0.9$  and  $25.9 \pm 1.4$  kcal/mol, respectively.





inflection point in the Arrhenius plot of this enzyme which had remained apparent after all other treatments examined. A unique value of 24 kcal/mol for the apparent activation energy is now obtained. This effect of treatment with phospholipase-A was not seen with cation activation of the enzyme as a discontinuity in the Arrhenius plot was still apparent under these conditions (Fig. 6). However, the difference in values between  $Ea_1$  and  $Ea_{11}$  was now smaller than usual, and the point of inflection in the plot could not be determined with as much confidence as before. The computed values for  $Ea_1$ ,  $Ea_{11}$  and  $T^\circ c$  were  $22.0 \pm 0.5$  kcal/mol,  $36.0 \pm 6.7$  kcal/mol and  $18.3 \pm 2.7^\circ C$ , respectively.

When the phospholipase-A treated preparations of  $(Na^+ + K^+)$ -ATPase were subsequently reincubated with phosphatidyl serine at a concentration of 3 mg phospholipid/mg of enzyme protein, the temperature-activity relationship which is shown in Fig. 7 was obtained.

The inflection point in the Arrhenius

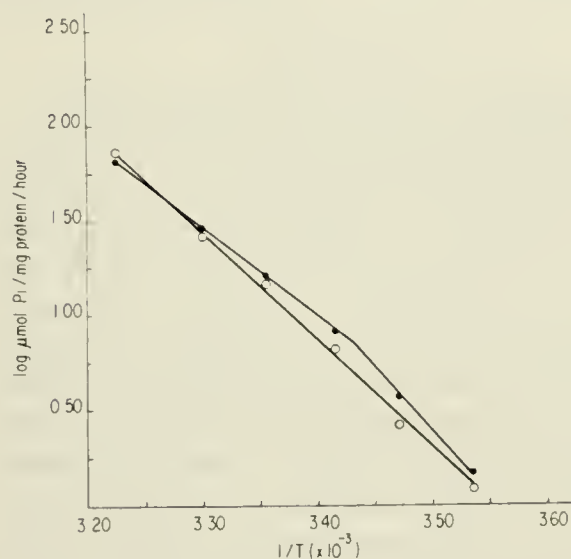


FIG. 6. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●—●) and ouabain inhibition (○—○) of membrane preparations of  $(Na^+ + K^+)$ -ATPase after mild treatment with phospholipase-A. There is no inflection point in the plot determined after ouabain inhibition, whereas the mean critical temperature ( $T^\circ c$ ) for four experiments after cation activation is  $18.3 \pm 2.7^\circ C$ . The computed values for  $Ea_1$  and  $Ea_{11}$  after cation activation are  $22.0 \pm 0.5$  and  $36.0 \pm 6.7$  kcal/mol, respectively. The unique value for  $Ea$  obtained after ouabain inhibition is  $24.0 \pm 3.9$  kcal/mol.

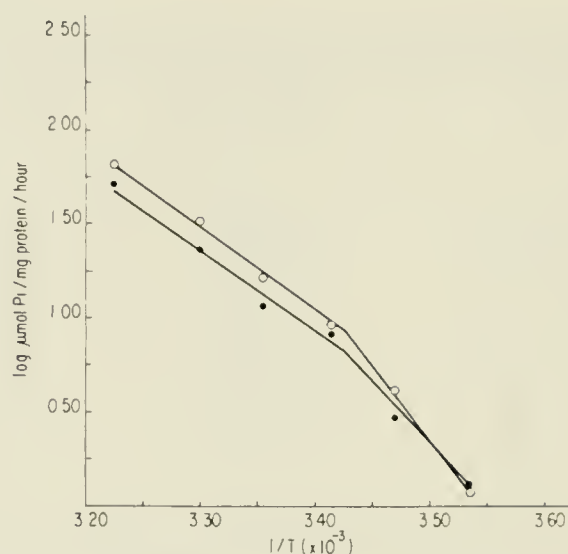


FIG. 7. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●—●) and ouabain inhibition (○—○) of membrane preparations of  $(Na^+ + K^+)$ -ATPase after mild treatment with phospholipase-A, followed by reincubation with phosphatidyl serine. Identical inflection points are determined under both experimental conditions at  $18.7 \pm 0.5^\circ C$  (mean of four experiments).

The computed values for  $Ea_1$  above  $T^\circ c$  are  $21.6 \pm 0.5$  and  $20.2 \pm 1.1$  kcal/mol for activation by cations and inhibition by ouabain, respectively. The values for  $Ea_{11}$  below  $T^\circ c$  are  $28.7 \pm 1.2$  and  $43.6 \pm 7.2$  kcal/mol, respectively.

plot previously obtained in the presence of  $10^{-4}$  M ouabain is regained (Figs. 1–5), thus confirming our earlier observation (12) and that of other workers (11, 13). The values for  $Ea_1$ ,  $Ea_{11}$  and  $T^\circ c$  which were computed under these experimental conditions, and in the presence of ouabain, were  $20.2 \pm 1.1$  kcal/mol,  $43.6 \pm 7.2$  kcal/mol and  $18.7^\circ \pm 0.5^\circ C$ , respectively. Conversely the effect of phosphatidyl serine upon cation activation of phospholipase-A treated  $(Na^+ + K^+)$ -ATPase is only slight, with very similar values for  $Ea_1$  and  $Ea_{11}$  being obtained before and after incubation with phosphatidyl serine (Table II). The apparent change in the critical temperature observed may be due to an excess of phosphatidyl serine in the system (24).

Therefore in order to detect any possible direct effect of this acidic phospholipid, a previously *untreated* enzyme preparation was also incubated with phosphatidyl serine. The temperature-activity relationship observed was not significantly



CATION ACTIVATION OF MEMBRANE ATPase

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TABLE II  
APPARENT ACTIVATION ENERGY OF CATION ACTIVATED ( $\text{Na}^+ + \text{K}^+$ )-ATPase AFTER VARIOUS TREATMENTS<sup>a</sup>

Conditions	No. of experiments	$Ea_i$ above $T^{\circ}\text{C}^b$	$Ea_{ii}$ below $T^{\circ}\text{C}^b$	$T^{\circ}\text{C}$
Untreated control	4	$15.4 \pm 0.7$	$27.5 \pm 1.9$	$16.7 \pm 1.6$
Sodium iodide	3	$15.4 \pm 1.1$	$27.0 \pm 1.0$	$20.6 \pm 4.1$
Deoxycholate	4	$17.5 \pm 2.1$	$30.4 \pm 5.9$	$18.7 \pm 1.4$
Nonidet P40	4	$15.6 \pm 0.6$	$25.6 \pm 1.0$	$19.5 \pm 1.2$
Phospholipase-C	4	$11.4 \pm 0.4$	$20.0 \pm 0.9$	$21.2 \pm 3.4$
Phospholipase-A	4	$22.0 \pm 0.5$	$36.0 \pm 6.7$	$18.3 \pm 2.7$
Phospholipase-A and phosphatidyl serine	4	$21.6 \pm 0.5$	$28.7 \pm 1.2$	$18.7 \pm 0.5$
Phosphatidyl serine	3	$17.0 \pm 0.7$	$30.7 \pm 1.7$	$16.2 \pm 1.3$

<sup>a</sup> Temperature-activity relationship determined by cation activation with 80 mM  $\text{Na}^+$  and 20 mM  $\text{K}^+$ , see Charnock and Post (21).

<sup>b</sup> Values for  $Ea_i$  and  $Ea_{ii}$  given as kcal/mol  $\pm$  standard error of the mean.

different from those seen with untreated controls (Fig. 1) and is therefore not shown.

In general all the experiments reported in this paper indicate that the activation of the enzyme preparations by  $\text{Na}^+$  and  $\text{K}^+$  ions is less influenced by change in temperature, than is the inhibition of the enzyme by ouabain. This overall effect is illustrated in Fig. 8.

DISCUSSION

The temperature-activity relationships of ( $\text{Na}^+ + \text{K}^+$ )-ATPase have been shown to be nonlinear over the range of 5–37°C, with the point of inflection at about 18°C. Above this critical temperature ( $T^{\circ}\text{C}$ ) the apparent activation energy ( $Ea_i$ ) of the overall hydrolysis of adenosine-triphosphate to adenosine-diphosphate and inorganic phosphate by this enzyme, is not greatly different whether enzyme activity is determined by its activation by the cations (sodium plus potassium) or by its inhibition by the cardiac glycoside ouabain in the presence of  $\text{Na}^+ + \text{K}^+$ . Conversely, there is a much greater difference between the apparent activation energies which are determined below the critical temperature ( $Ea_{ii}$ ) under these two experimental conditions; the mean value obtained by cation activation of untreated enzyme preparations being about 60% of that determined by ouabain inhibition. This decrease in activation energy is not

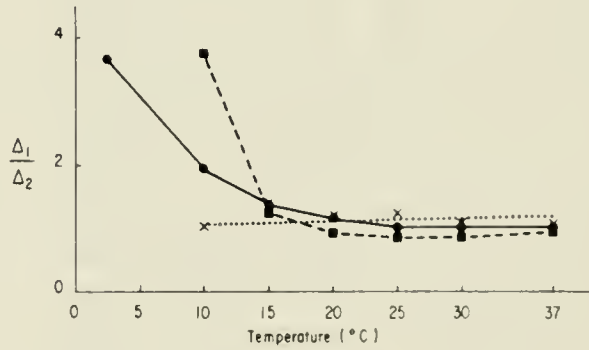


FIG. 8. The differential effect of temperature upon cation activation ( $\Delta_1$ ) and ouabain inhibition ( $\Delta_2$ ) of ( $\text{Na}^+ + \text{K}^+$ )-ATPase. (●—●) untreated enzyme preparation (values taken from Fig. 1). (×—×) after treatment with phospholipase-A (values taken from Fig. 6). (■—■) after treatment with phospholipase-A and reincubation with phosphatidyl serine (values taken from Fig. 7).

The data points are the mean specific activity of four enzyme preparations.

influenced by procedures known to partially remove lipids from such preparations (24). Apparently activation of ( $\text{Na}^+ + \text{K}^+$ )-ATPase by specific cations is much less influenced by changes in temperature, particularly below 18°C, than is its inhibition by cardiac glycosides. This suggestion is reinforced by the observed effect of treatment with NaI. This potentially chaotropic agent is known to disrupt lipid-protein and protein-protein interactions in biological membranes but does not solubilize their lipid components (26, 27). In these experiments, treatment





of ( $\text{Na}^+ + \text{K}^+$ )-ATPase preparations enhanced their specific activity but did not effect the apparent energy of activation of the enzyme in any way when its function was determined by inhibition by ouabain. However, when enzyme activity was determined by activation by cations, the transition temperature ( $T^\circ\text{C}$ ) was significantly changed by treatment with NaI, although the apparent activation energies were not altered.

Although further experiments will be required to more clearly establish the point, it is possible that the effect of NaI is more directly related to conformational changes in the protein moiety of the enzyme preparation than to altered phase transitions in the lipid moiety.

In this connection it is of great interest that *mild* treatment of our membrane preparations of ( $\text{Na}^+ + \text{K}^+$ )-ATPase with bee-venom phospholipase-A (12), has a profound effect upon the apparent activation energy of the process of ATP hydrolysis when determined by cardiac glycoside inhibition, but much less effect upon the process of ATP hydrolysis by this enzyme when determined by cation activation. Under conditions of cation activation, the nonlinearity of the temperature-activity relationships persists after phospholipase-A treatment, with an inflection point near  $18^\circ\text{C}$  and values of about 22 kcal/mol for  $Ea_1$  and 36 kcal/mol for  $Ea_{11}$  (Table II). When the apparent activation energy is measured by ouabain inhibition an inflection point cannot be determined under these conditions. The unique value for  $Ea$  which is now obtained between  $5^\circ$  and  $37^\circ\text{C}$  is  $24.0 \pm 3.9$  kcal/mol. In addition, when phospholipase-A treated preparations are incubated with phosphatidyl serine (the ratio of phospholipid to enzyme protein was 3:1), the cardiac glycoside-determined response becomes qualitatively similar to that seen with an untreated control preparation; that is, an inflection point is again apparent, and  $Ea_{11}$  is much greater than  $Ea_1$ .

Phosphatidyl serine has been reported to be preferentially bound to delipidated membranes (24). Apparently, reconstitution of phospholipase-A treated ( $\text{Na}^+ +$

$\text{K}^+$ )-ATPase by phosphatidyl serine restores both cardiac glycoside sensitivity and temperature dependence to the enzyme (6, 12, 14). *Mild* treatment with phospholipase-C does not produce this effect, although it seems probable that longer incubation of the enzyme with this lipase could reproduce the action of phospholipase-A.

In summary, this study has confirmed that the temperature-activity relationship of membrane-bound ( $\text{Na}^+ + \text{K}^+$ )-ATPase is nonlinear over the range  $5^\circ$ – $37^\circ\text{C}$  for the enzyme activity determined either by activation by cations or by inhibition with ouabain. However, the temperature dependence of ouabain inhibition is greater than that of cation activation, particularly below the inflection point of a temperature-activity plot. Mild lipolysis by phospholipase-A has little effect upon the cation activation of the enzyme at any temperature examined, but has a very marked effect in decreasing the apparent activation energy below  $18^\circ\text{C}$  when this is determined by inhibition of the enzyme by ouabain. The action of phospholipase-A can be overcome by reconstitution of the enzyme preparation with phosphatidyl serine.

These findings suggest that the lipid components of the membrane undergo a temperature-dependent phase transition at about  $18^\circ\text{C}$ , which in turn influences the activity of the ( $\text{Na}^+ + \text{K}^+$ )-ATPase enzyme protein. This cooperative effect is much more apparent when ouabain inhibition of the enzyme is examined rather than cation activation. The discriminating effect of phospholipase-A plus phosphatidyl serine suggest that a unique lipid component of the membrane, perhaps phosphatidyl serine in general but possibly even a phosphatidyl serine moiety at a special location in the membrane (24, 28), is particularly involved in imparting cardiac glycoside sensitivity and temperature dependence to ( $\text{Na}^+ + \text{K}^+$ )-ATPase. It seems much less probable that this suggested lipid moiety is involved in imparting cation activation to the system. Presumably it is this suggested lipid site which is greatly concerned with





the binding of cardiac glycosides, but not the binding of sodium or potassium ions to the membrane system in general.

#### ACKNOWLEDGMENTS

This work was supported by grants-in-aid from the Alberta Heart Foundation and the Medical Research Council of Canada.

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## APPENDIX III



APPENDIX III

Acetic acid, Glacial - Baker.

Acetic anhydride - Baker.

ATP-adenosine triphosphate, sodium. Grade 1 - Sigma.

Albumin, bovine serum - Sigma.

Calcium chloride - Fisher.

Chloroform - Fisher.

Cholesterol - Matheson, Coleman and Bell.

DOC-deoxycholic acid, sodium - Sigma.

DOC-<sup>14</sup>C deoxycholic acid, sodium - Amersham/Searle.

Dimyristoyl phosphatidyl choline - Serdary Research Laboratories.

1,4-Dioxane, purified - Fisher.

EDTA - ethylene diamine tetra acetic acid - Fisher.

Glycylglycine - Sigma.

L-Histidine - Sigma.

Lactic dehydrogenase, Type II - Sigma.

Magnesium sulfate - Fisher.

Mercaptoethanol - Sigma.

Methanol - Fisher.

$\beta$ -nicotinamide adenine dinucleotide, reduced form - Sigma.

Naphthalene - Fisher.





APPENDIX III (Cont'd)

Ouabain - Sigma.

PEP-phosphoenol pyruvate - Sigma.

Phosphatidyl serine - Serdary Research Laboratories.

PPL-A-phospholipase-A, bee venom - Sigma.

PP0(2,5-diphenyloxazole), Scintanalyzed - Fisher.

Potassium chloride - Baker.

Pyruvate kinase, Type II - Sigma.

Sodium chloride - Fisher.

SDS - sodium dodecyl sulfat - Sigma.

SD<sup>35</sup>S-sodium - NEN.

Sodium sulfate - Fisher.

Sucrose - BDH Chemicals.

Sulfuric acid - Baker.

Tris base - Schwarz/Mann.

Toluene, Scintanalyzed - Fisher.



APPEMDIX IV



#### APPENDIX IV

##### Treatment of membranes with deoxycholic acid (DOC).

The earlier work on  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  from this laboratory had been performed with a membrane preparation from rabbit renal cortex (see appendices I and II). As the ouabain binding experiments required larger amounts of enzyme than had hitherto been used, the rabbit kidney became an inadequate source of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . For this reason it was replaced by the ox brain.

The first DOC treatment applied to the brain membranes was adapted from the earlier studies that employed rabbit kidney membranes. A three fold improvement in enzyme specific activity was achieved, from 15 to 49  $\mu\text{moles Pi/mg protein/hr}$ , and this is shown in Table 1.

Further purification was attempted by extracting the membranes with 0.1% DOC at  $30^\circ$  after protection with ATP. The protection of enzymes with ATP in the presence of detergents is extensively used at present and its effectiveness is not in any doubt. In spite of this a clear understanding of this phenomenon at the molecular level has not yet been achieved. It is possible that a particular protein conformation may be involved. For example, if the ATP-enzyme complex sustains a certain conformation of the protein, then it is conceivable that specific phospholipids may also be protected against removal by the detergent. For this reason an extraction with DOC in the presence of  $\text{Na}^+$  and  $\text{Mg}^{++}$  was also attempted. The results are shown in Table 2.

It is noteworthy that the homogenisation procedure which was changed from a glass mortar with teflon pestle, to a polytron, has resulted in an increase in the specific activity of the untreated membranes.





Table 1.

	*Specific activity	%
Untreated	15	100
0.1% DOC, 4°, 10 min	49	327

Table 2.

Untreated	23	100
0.1% DOC, 4mMATP, 30°, 60 min	140	607
0.1% DOC, 4mMATP, 80mMNa, 2mM Mg, 30°, 60 min	39	168
0.2% DOC, 4mMATP, 80mMNa, 2mMMg, 30°, 60 min	1	5

Table 3. Time study using 0.05% DOC, 4mM ATP, 80mM Na, 2mM Mg, at 30°.

0 min	20	100
2	32	165
4	37	185
6	36	180
8	44	220
10	39	195

Table 4. Time study using 0.1% DOC, 4mM ATP, 80mM Na, 2mM Mg, at 30°.

0 min	19	100
10	126	652
20	114	590
30	94	488
40	119	616
50	118	610

\*Specific activity given in  $\mu$ molesPi/mg protein/hour.



Extraction of the membranes with 0.1% DOC at 30° markedly improves the purification process, resulting in a six-fold increase in specific activity. This is not the case when the cations  $\text{Na}^+$  and  $\text{Mg}^{++}$  are present. With 0.1% DOC less than a two-fold improvement results and if the DOC is increased to 0.2% then the enzyme activity is lost. Thus it appears that when cations are present the enzyme is more labile, and the optimal concentrations of DOC and/or exposure time, is altered.

Table 3 shows a time study of the effects of 0.05% DOC on membranes pretreated with 4mM ATP in the presence of 80mM  $\text{Na}^+$  and 2mM  $\text{Mg}^{++}$ . From the data in this table it is apparent that this low concentration of DOC again produces a relatively small increase in the specific activity of the enzyme. Only a two-fold increase is obtained after an exposure time of 8 min. On comparison with the data shown in Table 2, it seems possible that a better purification might be achieved with 0.1% DOC and a shorter exposure time. This possibility was examined, and the results are shown in Table 4.

The data show that about a six-fold purification is the maximum that can be achieved under these conditions, and it is attained within 10 min of addition of the detergent. Quite clearly the presence or absence of cations, the concentration of DOC and the exposure time are not the only factors controlling the degree of purification of these membranes. Subsequent purification employing 0.1% DOC and 4mM ATP, showed little change after 30 to 35 min and up to 60 min. Since the attainment of ideal conditions for purification was not essential to routine procedure hereafter was a 40-minute exposure to 0.1% DOC at 30° after the enzyme had been protected with 4mM ATP.



## APPENDIX V





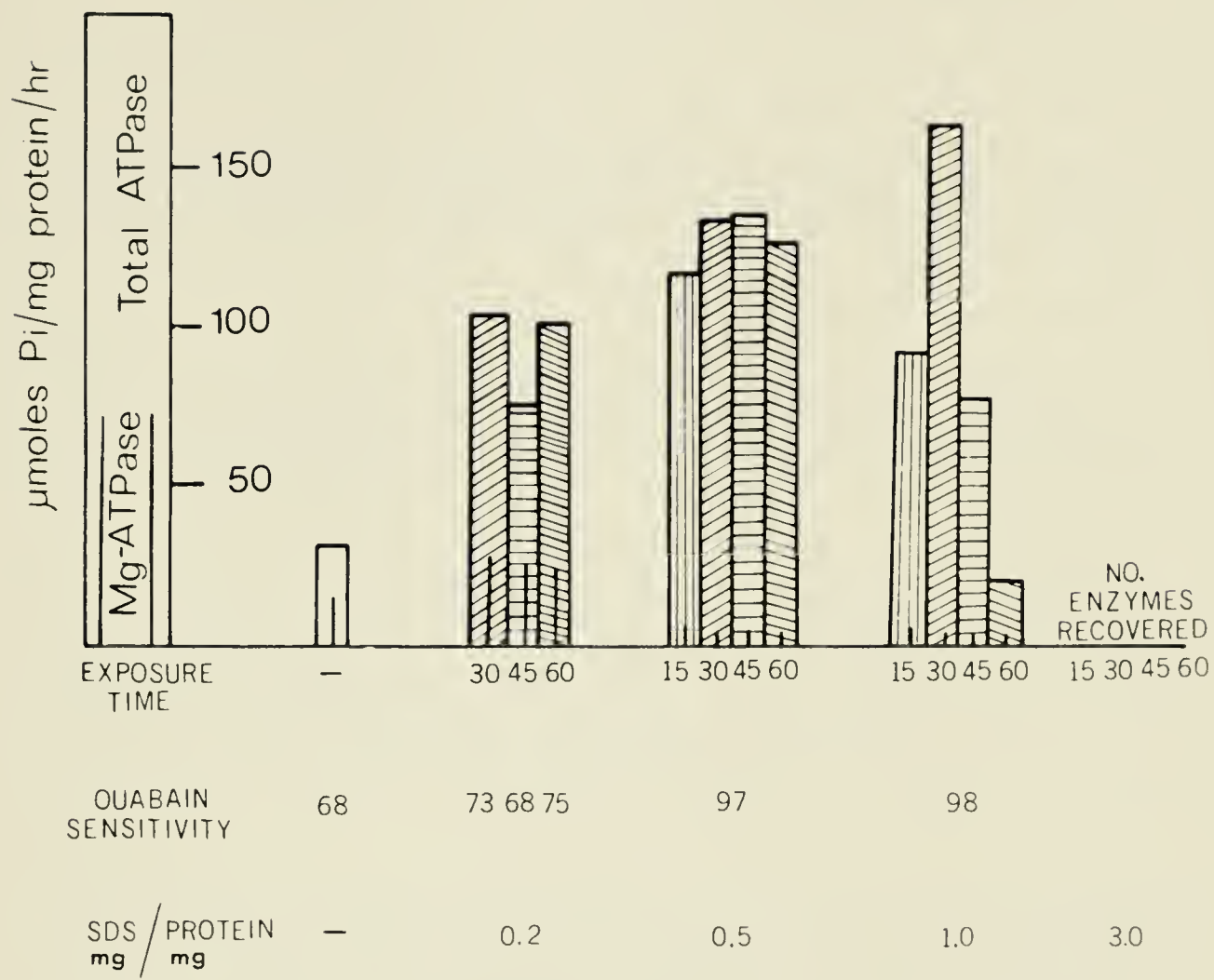
## APPENDIX V

The histogram shows the effect of two variables on  $(\text{Na}^+ + \text{K}^+)$ -ATPase activity, namely SDS/protein ratio and the exposure time. The following observations can be drawn.

1. There is a gradual increase in the specific activity of the enzyme in response to the concentration of SDS.
2. At the higher SDS concentrations, solubilization of the protein follows purification of the  $(\text{Na}^+ + \text{K}^+)$ -ATPase. This results in a decreased purity as well as a loss of recovery.
3. Optimal purification may result on treatment of the enzyme with 0.1% SDS, but at this concentration the exposure time becomes very critical. Therefore as a routine procedure it is unsuitable.
4. Treatment of the enzyme with 0.05% SDS results in about a four-fold purification over a broad exposure time of about 30 to 45 minutes.

From this study the routine SDS purification procedure employed a 30-minute exposure of 1mg protein/ml to 0.05% solution of SDS. Usually a five-fold purification was observed but the value varied from not less than 4-fold to a use of 8-fold.







APPENDIX VI





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## VARIATION IN SENSITIVITY OF THE CARDIAC GLYCOSIDE RECEPTOR CHARACTERISTICS OF $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ TO LIPOLYSIS AND TEMPERATURE

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### Summary

1. The rate of binding of  $[^3\text{H}]$ ouabain to untreated membrane preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  is a temperature-dependent process displaying a thermal transition close to  $25^\circ\text{C}$ . The apparent energies of activation which can be calculated above and below this transition are similar to, but not identical with, those previously reported for activation of the enzyme by cations.

2. Treatment of the enzyme preparation with detergents or lipolysis with phospholipase A eliminates the thermal transition resulting in linear Arrhenius plots.

3. The number of sites available for  $[^3\text{H}]$ ouabain binding is not temperature dependent as the amount of  $[^3\text{H}]$ ouabain bound at equilibrium is not changed between 10 and  $37^\circ\text{C}$ .

4. Treatment of the enzyme with phospholipase A results in time-dependent changes in the number of binding sites for  $[^3\text{H}]$ ouabain at equilibrium.

5. Treatment of the membrane enzyme preparations with detergents reveals additional  $[^3\text{H}]$ ouabain binding sites which are extremely sensitive to lipolysis with phospholipase A.

6. There are a number of  $[^3\text{H}]$ ouabain binding sites which remain resistant to lipolysis by phospholipase A in either untreated or detergent-treated membrane preparations.

7. It is suggested that  $[^3\text{H}]$ ouabain binding sites exist in the membrane in at least two different environments, one of which is resistant the other sensitive to attack by phospholipase A.

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### Introduction

There is a continuing interest in the mechanism of action of the cardiac glycosides as this potent group of pharmacologic agents has been in continuous



therapeutic use since the pioneering work of Withering in 1785 [1]. For example, since it was first suggested a decade ago that ouabain blocks cation transport by inhibiting the turnover of a phosphorylated intermediate of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  [2–4], there have been numerous studies of the interaction of that cardiac glycoside with particulate preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  obtained from a wide variety of tissues and species [5–7]. In confirmation of the earlier reports of Schwartz and his colleagues [8,9], Erdmann and Schoner [10,11] have recently produced strong evidence that the characteristics of [ $^3\text{H}$ ]ouabain binding to membrane preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  reflect the properties of a cardiac glycoside receptor.

Studies of the effect of temperature upon both cation activation and ouabain inhibition of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  in our laboratory suggest that the lipid membranes containing this enzyme exert a differential effect at the sites of cation activation and cardiac glycoside inhibition [12,13]. This paper describes a series of experiments in which this possibility is explored more directly by examining the effect of temperature upon the binding of [ $^3\text{H}$ ]ouabain to preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  following a variety of treatments known to influence membrane lipids.

## Materials and Methods

*Enzyme preparation.* Ouabain-sensitive  $(\text{Na}^+ + \text{K}^+)\text{-activated ATPase}$  (EC 3.6.1.3) was prepared from both fresh or frozen beef brain by the general procedure of Charnock and Post [14]. Homogenization was carried out at  $4^\circ\text{C}$  in the medium described previously [12] using either (a) four strokes of a teflon-glass homogenizer, or (b) one 10 s pulse in a Polytron Homogenizer fitted with a PT-20 generator and operated at setting 8. After removal of the cellular debris by centrifugation at  $1000 \times g$  for 15 min in a refrigerated Sorvall RC2-B centrifuge fitted with a SS34 rotor, mitochondrial particles were removed by centrifuging at  $9000 \times g$  for 20 min. A “heavy microsomal” pellet was then isolated by centrifugation at  $46000 \times g$  for 30 min. This pellet was washed twice by resuspension and sedimentation in buffer (20 mM Tris  $\cdot$  HCl/1 mM EDTA at pH 7.6). The washed pellets were resuspended in this buffer and stored at  $-20^\circ\text{C}$  after rapid freezing in liquid  $\text{N}_2$ . The protein content of these microsomal suspensions, which was determined by the method of Lowry et al. [15], ranged from 2 to 8 mg per ml.

Membrane enzyme preparations obtained by both method a and b were treated with 0.1% deoxycholic acid for 10 min at  $4^\circ\text{C}$ ; with 0.1% deoxycholic acid in the presence of 3 mM ATP for 30 min at  $30^\circ\text{C}$ ; with 0.05% deoxycholic acid and 2 mM ATP in the presence of 5 mM  $\text{MgSO}_4$  and 80 mM NaCl for 5 min at  $30^\circ\text{C}$ ; and with 0.1% sodium dodecyl sulfate plus 4 mM ATP for 30–60 min at  $30^\circ\text{C}$  as indicated in the text. After each of these treatments the detergent-extracted membranes were sedimented at  $46000 \times g$  for 60–120 min and the pellets washed twice by resuspension in buffer.

The procedures for treatment of the enzyme preparations with phospholipase A and subsequent reconstitution was phosphatidylserine were adopted from Imai and Sato [16] and have been described previously [12]. The ratio of phospholipase A to enzyme preparation were determined by preliminary





experiments which monitored the extent of lipolysis by continuous titration of free fatty acid release. Maximum lipolysis usually occurred within 10 min of addition of phospholipase A. Further addition of phospholipase A did not result in further liberation of free fatty acid. Usually this procedure yielded a product having a specific ouabain-sensitive ATPase activity from 20 to 30% of the untreated control preparation. Variations from this time of lipolysis are described in the text.

*[<sup>3</sup>H]Ouabain binding studies.* Binding studies were performed in an incubation medium of 100 mM glycylglycine, 2 mM MgSO<sub>4</sub>, 80 mM NaCl, 2 mM ATP and 0.2 mM H<sub>4</sub>EDTA adjusted to pH 7.6 with 1 M Tris/base. Except for some preliminary experiments which are discussed later in the text, the final concentration of [<sup>3</sup>H]ouabain was  $5 \cdot 10^{-7}$  M. The specific radioactivity of the [<sup>3</sup>H]-ouabain was maintained between 300 and 400 dpm per pmol ouabain.

The bound [<sup>3</sup>H]ouabain was determined by a rapid Millipore filtration method similar to that employed by others [17,18]. At rapid intervals (as short as 5 s at higher temperatures) 1-ml aliquots were removed from the incubation vessel and filtered on 0.8  $\mu$ m Millipore filters. Protein retention was virtually complete under all experimental conditions employed, as analysis of filtrates revealed no detectable protein. Assay for ATPase activity in the filtrates also failed to reveal any enzyme activity.

To negate non-specific drug binding to the filters, they were prewashed with 2 ml of a wash solution whose composition was identical to the binding medium but without ATP or radioactivity. The temperature of the binding medium and its respective wash solution was identical. After the initial filtration step, two washes of 2 ml each ensured removal of unbound drug. Increasing the number of washes did not reduce the levels of [<sup>3</sup>H]ouabain bound.

1-ml aliquots of the incubation mixture were removed for binding analysis at various times after the addition of the enzyme preparations. Binding times were taken to the time the aliquot was applied to the filter. At 37°C the aliquots were removed at 5-s intervals; at lower temperatures the intervals were increased until at 9°C the whole operation took about 250 s. However, at all temperatures samples were removed at much longer intervals (up to 60 min), so that equilibrium levels of [<sup>3</sup>H]ouabain could be obtained.

Rates of [<sup>3</sup>H]ouabain binding were determined from the slopes of regression analyses of the levels of bound drug, using the Olivetti program No. 681009. Assays were always in duplicate or more. Individual assays varied less than  $\pm 5\%$ , and the mean values were reproducible upon repeated assay of samples stored at  $-20^{\circ}\text{C}$ .

The filters were dried in air, disintegrated in 1 ml of methanol and dissolved in 10 ml of 13.5% (v/v) toluene-dioxane fluor and counted to 3% error in a Beckmann LS-100 liquid scintillator.

*ATPase activity measurement.* All enzyme preparations used in the [<sup>3</sup>H]-ouabain binding study were routinely assayed for both ouabain-sensitive and ouabain-insensitive ATPase activity under similar conditions to the binding studies.

Enzyme activity was measured by a coupled optical assay system using a Gilford 2400 recording spectrophotometer equipped with a jacketed ethylene glycol/water constant temperature bath [9,19,20]. The temperature of the





spectrophotometer cell was controlled to  $\pm 0.2^\circ\text{C}$ . Reactions were performed in 100 mM glycylglycine (pH 7.6) containing 2 mM  $\text{MgSO}_4$ , 80 mM NaCl, 20 mM KCl with 250 mM sucrose added to prevent protein settling. The assay ingredients included 3.14 mM phosphoenolpyruvate (sodium salt) and 64 units of pyruvate kinase, 19 units lactate dehydrogenase and 0.2 mg NADH in a final volume of 3 ml. The enzyme preparation was added to the reaction cuvette and allowed to thermally equilibrate before the reaction was initiated by the addition of ATP to a final concentration of 0.4 mM. Monitoring of NADH oxidation at 340 nm commenced immediately after mixing of the cuvette contents.  $\text{Mg}^{2+}$ -ATPase activity was followed in the presence of 0.4 mM ouabain, and was subtracted from the total ATPase activity obtained in the presence of  $\text{Mg}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$  to give the ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase activity.

*Determination of activation energies.* The effect of temperature on ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-dependent ATPase activity, and on the rate of [ $^3\text{H}$ ]ouabain binding to these enzyme preparations was determined by a procedure described previously [21]. The data which could be displayed as Arrhenius plots, were further analyzed by the technique of Bogartz [22] for fitting either a single or two intersecting lines, utilizing an APL/360 computer program developed in this laboratory [21]. This analysis yields values for both the apparent activation energies and the critical temperature of the system.

*Materials.* ATP (disodium salt), glycylglycine, L-histidine (free base), bee venom phospholipase A, phosphoenolpyruvate (sodium salt), pyruvate kinase, lactate dehydrogenase and NADH were all obtained from the Sigma Chemical Co.; [ $^3\text{H}$ ]ouabain from New England Nuclear, sucrose (ANALAR grade) from British Drug House Ltd., (Chemical Division); and phosphatidylserine (bovine brain) from Serdary Research Laboratories (Montreal, Quebec).

## Results

### *Preliminary experiments*

Although it is well known that two different sets of experimental conditions lead to optimal binding of [ $^3\text{H}$ ]ouabain to ( $\text{Na}^+ + \text{K}^+$ )-ATPase [20,23–27], we chose to examine [ $^3\text{H}$ ]ouabain binding under those conditions which had been previously shown to lead to optimal enzyme phosphorylation, i.e. the presence of ATP,  $\text{Mg}^{2+}$  and  $\text{Na}^+$  [3,4,7,28–30]. In addition, Erdmann and Schoner [10, 11,31] have reported that both maximum drug receptor occupancy and inhibition of ( $\text{Na}^+ + \text{K}^+$ )-ATPase activity occur at a ouabain concentration very near  $5 \cdot 10^{-7}$  M; although many other workers have shown that the  $K_i$  for ouabain inhibition of ( $\text{Na}^+ + \text{K}^+$ )-ATPase activity is  $5 \cdot 10^{-6}$  M [2,29,30].

We therefore commenced our study by examining [ $^3\text{H}$ ]ouabain binding under these reported “optimising” conditions. From preliminary experiments at  $37^\circ\text{C}$  we found that in agreement with the reports of Taniguchi and Iida [32, 33], the rate of [ $^3\text{H}$ ]ouabain binding only remains linear for about the first 30 s of the experiment; that at this temperature an equilibrium level of bound ouabain is established after about 2 min of incubation; and that this level remains constant thereafter. In addition the binding of [ $^3\text{H}$ ]ouabain in the absence of ATP and other activating ligands is negligible compared to that in the presence of both ATP and  $\text{Na}^+$ . However, in contrast to the findings of Erd-



mann and Schoner [11], we found that the maximum rate of binding was not achieved at  $5 \cdot 10^{-7}$  M [ $^3$ H]ouabain. With ouabain concentrations higher than  $1 \cdot 10^{-6}$  M, the rate of drug binding was too fast to be measured experimentally, even by our rapid filtration method, since at  $37^\circ\text{C}$  equilibrium levels of binding were reached within 5 s of protein addition. This effect of drug concentration was apparent over the range of enzyme protein concentrations examined (90–240  $\mu\text{g}$  protein/ml). We therefore continued to examine the rate of ouabain binding at a concentration of  $5 \cdot 10^{-7}$  M which, although not maximal was technically convenient.

#### *Effect of temperature and lipolysis on the rate of ouabain binding*

The effect of temperature upon the rate of [ $^3$ H]ouabain binding was examined in experiments described in Fig. 1 where it can be seen that the rate of drug binding decreases with decreasing temperature. It is also evident that the linearity of the rate of binding was maintained under the conditions of our experiments. The mean rates of [ $^3$ H]ouabain binding that can be obtained from these experiments are typical of the values which were used to construct the Arrhenius plots described later in this paper.

All studies of the rate of [ $^3$ H]ouabain binding were conducted within the linear time period of drug uptake.

In Fig. 2 we give the results of more than 40 experiments using  $(\text{Na}^+ + \text{K}^+)$ -

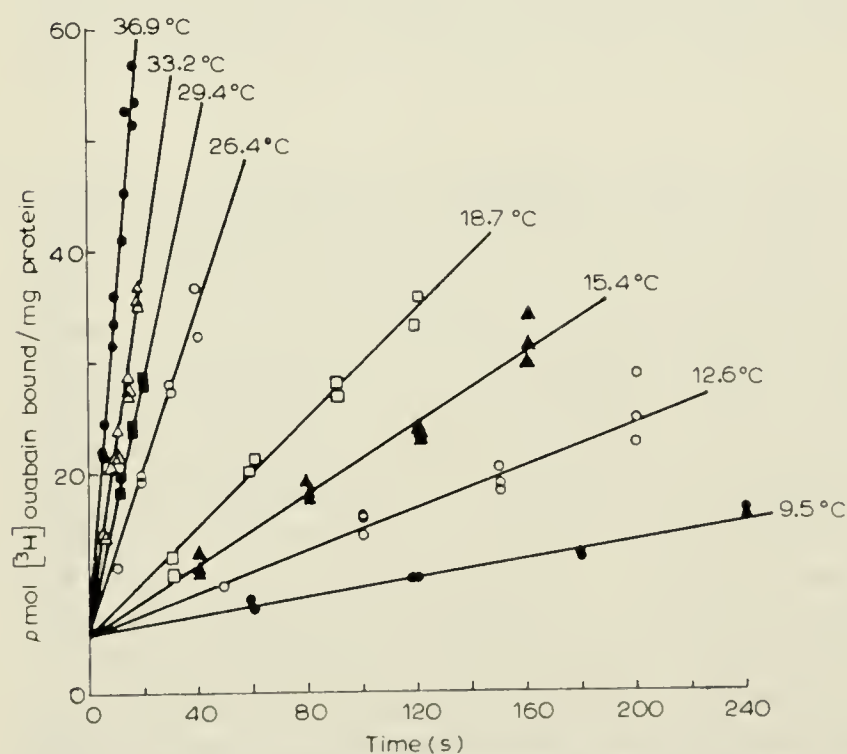


Fig. 1. The effect of temperature on the binding of [ $^3$ H]ouabain to an untreated membrane preparation of  $(\text{Na}^+ + \text{K}^+)$ -ATPase. The enzyme preparation had a specific activity of  $16.5 \mu\text{mol P}_i/\text{mg}$  protein per h; 82% of the total ATPase activity of the preparation was inhibited by 0.4 mM ouabain. The protein concentration was  $107 \mu\text{g}/\text{ml}$ . The rate of [ $^3$ H]ouabain binding was determined from the slope of the line obtained at each experimental temperature, using the Olivetti programme No. 681009. The mean initial rates determined in this experiment were from  $9.5$  to  $36.9^\circ\text{C}$ , respectively: 0.05, 0.08, 0.13, 0.20, 0.59, 0.89, 1.24 and  $1.95 \text{ pmol } [^3\text{H}]\text{ouabain}/\text{mg}$  protein per s. Standard error of the means of each temperature were always  $<10\%$ . Assays were in duplicate or triplicate.





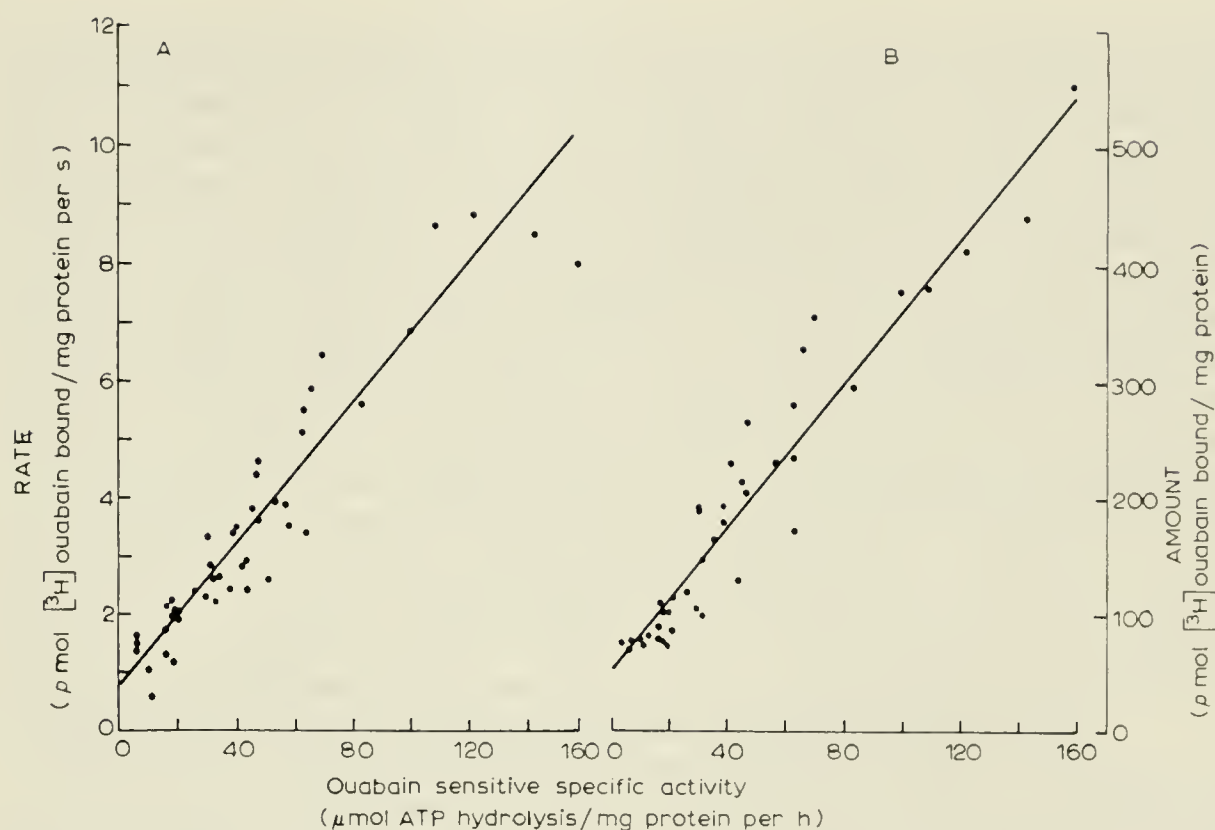


Fig. 2. Relationship of the specific activity of ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase with the rate of [ $^3\text{H}$ ]-ouabain binding (Panel A) and the amount of [ $^3\text{H}$ ]-ouabain bound at equilibrium (Panel B). All experiments were at  $37^\circ\text{C}$ . Enzyme preparations of widely different specific activity were obtained from untreated preparations and following the various detergent extractions with deoxycholic acid and sodium dodecyl sulfate described under Materials and Methods. Rates were determined from aliquots taken during the first 30 s after the addition of the enzyme. Each point represents a single enzyme preparation, assays were in duplicate or triplicate. Amounts of [ $^3\text{H}$ ]-ouabain binding at equilibrium were determined after 10 min incubation at  $37^\circ\text{C}$  and are the means  $\pm$  S.E. of duplicate assays.

ATPase preparations of widely variable specific activity obtained from both untreated and detergent-treated preparations. The relationship between the rate of [ $^3\text{H}$ ]-ouabain bound at  $37^\circ\text{C}$  and the specific activity of ouabain-sensitive ( $\text{Na}^+ + \text{K}^+$ )-ATPase activity at this temperature is shown in Panel A. It is clear that the rate of [ $^3\text{H}$ ]-ouabain binding increases with increasing specific activity of these preparations. The correlation shown is significant at the 0.05 level or better. Panel B gives the relationship between the amount of [ $^3\text{H}$ ]-ouabain bound at equilibrium and the specific activity of ( $\text{Na}^+ + \text{K}^+$ )-ATPase at  $37^\circ\text{C}$ . It is again evident that a good correlation is obtained between these latter parameters with significance at the 0.05 level or better. Because our data was obtained from both control and detergent-treated preparations, our findings both confirm and extend the earlier reports of Erdmann and Schoner [10,11].

From the information obtained in these experiments we were able to examine the effect of temperature upon [ $^3\text{H}$ ]-ouabain binding under conditions of ligand, drug and enzyme protein concentration shown to give rates of binding which were as close to the initial rates as we were able to determine by our procedure.

The experimental data obtained from a series of experiments with untreated enzyme preparations and after detergent extractions with deoxycholic acid under various conditions was first displayed as Arrhenius plots and then values





for the apparent energies of activation above and below the transition temperature were calculated [21,22]. The numerical values for these parameters are given in Table I which shows that in four experiments using untreated beef brain enzymes prepared by limited teflon-glass homogenization (Method a), it is possible to construct a non-linear Arrhenius plot for [ $^3\text{H}$ ]ouabain binding which can be described by two straight lines intersecting at a transition temperature ( $T$ ) of  $25.4 \pm 2.0^\circ\text{C}$ . Calculation of the mean apparent energies of activation above ( $E_{a,I}$ ) and below ( $E_{a,II}$ ) the critical temperature yields values of  $18.5 \pm 2.1$  and  $29.1 \pm 1.3$  kcal/mol, respectively. Comparison of these mean values by the paired " $t$ " test indicates statistical significance at the 0.01 level.

The mean data from another group of six experiments using untreated beef brain enzymes but prepared by Polytron disintegration (Method b) also yields a non-linear Arrhenius plot with an identical value for the transition temperature of  $24.6 \pm 1.40^\circ\text{C}$ . Although the mean values for  $E_{a,I}$  and  $E_{a,II}$  which are derived from this plot ( $20.9 \pm 0.33$  and  $25.7 \pm 0.86$  kcal/mol, respectively) are not as widely different from each other as are those seen in the previous group, comparison demonstrates statistical significance at the 0.001 level.

Mild detergent extraction of these two apparently similar enzyme preparations with 0.1% deoxycholic acid at  $4^\circ\text{C}$  for 10 min reveals some differences in these materials. With enzyme preparations prepared by method a, there are some obvious quantitative changes following extraction, as the mean values for  $E_{a,I}$  and  $E_{a,II}$  are now  $11.7 \pm 1.36$  and  $21.8 \pm 2.2$  kcal/mol, respectively. The thermal transition in the Arrhenius plot of [ $^3\text{H}$ ]ouabain binding is still apparent, although the mean transition temperature is now somewhat higher than before. ( $T$ ,  $29.0 \pm 1.3^\circ\text{C}$ ). The non-linearity of the temperature dependence of [ $^3\text{H}$ ]ouabain binding has remained unchanged.

On the other hand, when enzyme preparations prepared by method b were extracted with deoxycholic acid it was found that the mean value for  $E_{a,I}$  ( $20.1 \pm 1.35$  kcal/mol) was no longer significantly different ( $P > 0.40$ ) from the mean value for  $E_{a,II}$  ( $22.1 \pm 1.59$  kcal/mol). That is this mild detergent extraction of these beef brain preparations has given a product which no longer clearly displays a marked thermal transition for [ $^3\text{H}$ ]ouabain binding. Preparations obtained by the presumably less disruptive method a are more resistant to alteration by this form of detergent treatment than are preparations obtained by method b.

In addition, Table I also shows the results obtained by treatment of beef brain enzymes (prepared by method b) with deoxycholic acid at higher temperatures as well as treatment with sodium dodecyl sulfate or phospholipase A. All these more vigorous procedures yield data which can best be described by linear Arrhenius plots, as the Bogartz [22] analysis does not reveal either statistically significant differences in the activation energies which could be calculated for  $E_{a,I}$  and  $E_{a,II}$ , or meaningful values for the transition temperatures. It is of considerable interest that incubation of the enzyme preparation with phosphatidylserine after treatment with phospholipase A did not produce a non-linear temperature dependence for [ $^3\text{H}$ ]ouabain binding. Clearly both more vigorous extraction of ( $\text{Na}^+ + \text{K}^+$ )-ATPase with detergents and treatment with phospholipase A gives ratios of  $E_{a,I} : E_{a,II}$  which are close to unity, that is, plots which are best described as linear.



TABLE I  
APPARENT ACTIVATION ENERGIES CALCULATED FROM THE RATE OF [<sup>3</sup>H]OUABAIN BINDING TO VARIOUS (Na<sup>+</sup> + K<sup>+</sup>)-ATPase PREPARATIONS AT DIFFERENT TEMPERATURES

	Method	n	$E_{a,I} \pm S.E.$ (kcal/mol)	$E_{a,II} \pm S.E.$ (kcal/mol)	$\frac{E_{a,I}}{E_{a,II}}$	$T \pm S.E.$ (°C)	$\frac{E_{a,I} \text{ vs. } E_{a,II}}{P}$
Untreated	a	4	18.5 ± 2.10	29.1 ± 1.30	0.64	25.4 ± 2.00	<0.01
Deoxycholic acid (4° C)	a	7	11.7 ± 1.36	21.8 ± 2.20	0.54	29.0 ± 1.30	<0.01
Untreated	b	6	20.9 ± 0.33	25.7 ± 0.86	0.81	24.6 ± 1.40	<0.001
Deoxycholic acid (4° C)	b	3	20.1 ± 1.35	22.1 ± 1.59	0.91	27.2 ± 8.26	>0.40
Deoxycholic acid + ATP (30° C)	b	6	20.4 ± 0.56	21.9 ± 0.37	0.93	43.7 ± 3.06	>0.05
Deoxycholic acid + ATP + Mg <sup>2+</sup> + Na <sup>+</sup> (30° C)	b	5	22.8 ± 0.37	21.5 ± 1.79	1.06	9.5 ± 2.25	>0.50
Sodium dodecyl sulfate + ATP (30° C)	b	3	21.9 ± 1.84	21.4 ± 1.06	1.02	3.15 ± 3.41	>0.80
Deoxycholic acid (4° C) + phospholipase A *	b	3	26.5 ± 2.43	25.8 ± 2.31	1.03	-27.0 ± 12.4	>0.80
Deoxycholic acid (4° C) + phospholipase A * + phosphatidylserine	b	2	22.2 ± 0.80	24.3 ± 6.25	0.91	40.2 ± 36.7	>0.70

\* 5 units phospholipase A for 5 min at 37° C.

TABLE II  
APPARENT ENERGIES OF ACTIVATION CALCULATED FROM THE RATE OF ATP HYDROLYSIS BY OUABAIN-SENSITIVE (Na<sup>+</sup> + K<sup>+</sup>)-ATPase PREPARATIONS AT DIFFERENT TEMPERATURES

	Method	n	$E_{a,I} \pm S.E.$ (kcal/mol)	$E_{a,II} \pm S.E.$ (kcal/mol)	$\frac{E_{a,I}}{E_{a,II}}$	$T \pm S.E.$ (°C)	$\frac{E_{a,I} \text{ vs. } E_{a,II}}{P}$
Untreated	a	9	18.1 ± 1.10	37.0 ± 3.40	0.49	20.0 ± 1.00	<0.001
Deoxycholic acid (4° C)	a	6	21.8 ± 0.51	49.2 ± 3.90	0.44	17.8 ± 0.80	<0.001
Untreated	b	4	14.6 ± 1.95	29.8 ± 0.30	0.49	20.9 ± 1.31	<0.001
Deoxycholic acid (4° C)	b	4	19.7 ± 1.89	41.8 ± 3.08	0.47	17.9 ± 0.93	<0.001
Deoxycholic acid + ATP (30° C)	b	5	16.4 ± 0.80	45.4 ± 5.40	0.36	16.6 ± 1.83	<0.001
Deoxycholic acid + ATP + Mg <sup>2+</sup> + Na <sup>+</sup> (30° C)	b	3	15.1 ± 0.93	32.8 ± 5.30	0.46	19.2 ± 1.55	<0.05
Sodium dodecyl sulfate + ATP (30° C)	b	3	13.6 ± 1.68	30.2 ± 1.20	0.45	20.0 ± 1.27	<0.01
Deoxycholic acid (4° C) + phospholipase A *	b	3	26.9 ± 1.30	20.3 ± 4.20	1.32	30.8 ± 5.00	<0.20
Deoxycholic acid (4° C) + phospholipase A * + phosphatidylserine	b	2	17.6 ± 2.10	35.9 ± 3.45	0.49	22.7 ± 2.40	<0.05

\* 5 units phospholipase A for 5 min at 37° C.





By contrast, Table II gives the data for the temperature dependence of ATP hydrolysis by these enzyme preparations which were obtained in paired experiments. As we have demonstrated previously [12,13], there is a marked non-linear response to temperature with all untreated and treated preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  except following lipolysis with phospholipase A. Again we have been able to demonstrate that this effect of phospholipase A on the rate of ATP hydrolysis can be overcome by reconstitution of the treated enzyme preparations with phosphatidylserine [12,13]. However, under conditions of our experiments (3 mg phosphatidylserine/mg protein at 37°C for 10 min) reconstitution of phospholipase A-treated enzyme preparations with phosphatidylserine only restored the non-linearity to the Arrhenius plots for ATP hydrolysis and not to those for  $[^3\text{H}]\text{ouabain}$  binding (cf. Tables I and II). Nevertheless, it should be noted that the ratio of  $E_{a,I} : E_{a,II}$  which is obtained for  $[^3\text{H}]\text{ouabain}$  binding after treatment with phosphatidylserine shows a tendency towards non-linearity.

We can conclude from these studies of the effect of temperature on the rate of  $[^3\text{H}]\text{ouabain}$  binding to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  that this characteristic of the drug-receptor interaction is lipid dependent, and is more susceptible to change by detergent extraction than is the hydrolysis of the substrate by the enzyme. Apparently membrane lipids are involved in both processes but our preliminary attempts at reconstitution with phosphatidylserine imply that different membrane lipids are associated with these different characteristics of the enzyme receptor system.

#### *Effect of temperature and lipolysis on the amount of ouabain binding*

The effect of temperature on the amount of  $[^3\text{H}]\text{ouabain}$  which was bound to the enzyme preparation at equilibrium was also examined. The results of a study using untreated enzyme preparations is given in Table III. At true equilibrium temperature does not affect the amount of drug which can be bound; that is, temperature does not alter the number of ouabain receptors available for binding.

TABLE III

EQUILIBRIUM LEVELS OF OUABAIN BOUND TO BEEF BRAIN  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  AT VARIOUS TEMPERATURES

Binding reactions were started by the addition of protein to a final concentration of 225  $\mu\text{g/ml}$  and terminated by filtration after 30 min, except for the lowest temperature \* where equilibrium was not reached until 60 min after protein addition. The concentration of ouabain was  $5 \cdot 10^{-7}$  M in all experiments. The values given are the means  $\pm$  S.D. of four experiments.

Temperature (°C)	$[^3\text{H}]\text{ouabain}$ bound pmol/mg protein $\pm$ S.D.
36.8	87 $\pm$ 5.7
33	90.6 $\pm$ 2.3
29.3	85 $\pm$ 2.5
26.5	94.6 $\pm$ 5.2
18.5	94.3 $\pm$ 5.2
15.3	89 $\pm$ 3.6
12	85 $\pm$ 4.8
9.3	93.2 $\pm$ 4.2 *





However, it is possible to alter the number of ouabain binding sites under certain experimental conditions. For example, although mild treatment of the enzyme preparations with deoxycholic acid at 4°C only leads to a relatively small increase in the specific activity of the enzyme and in the amount of [<sup>3</sup>H]-ouabain bound at equilibrium, more pronounced detergent treatment with either deoxycholic acid or sodium dodecyl sulfate at 30°C in the presence of ATP produces a marked increase in both enzyme specific activity and the amount of [<sup>3</sup>H]ouabain bound at equilibrium (Table IV). Apparently, additional binding sites are exposed after detergent extraction, indicating that lipid-lipid or lipid-protein interactions previously prevented ready access of [<sup>3</sup>H]-ouabain to a potential binding site. It is important to note from the data in Fig. 2 that the increase in [<sup>3</sup>H]ouabain binding which follows treatment with detergents is proportional to the increase in ouabain-sensitive (Na<sup>+</sup> + K<sup>+</sup>)-ATPase which also occurs after this treatment.

In contrast to the increase in the amount of [<sup>3</sup>H]ouabain bound after detergent extraction, Taniguchi and Iida [32,33] have reported that treatment of the enzyme with phospholipase A resulted in a loss of activity and a reduction in the initial rate of [<sup>3</sup>H]ouabain binding. These workers also reported that the "binding capacities" of the ouabain binding site showed no remarkable change as a consequence of treatment with phospholipase A.

In our experiments we found that the effects of phospholipase A varied with both the time of lipolysis and with pretreatment of the enzyme with detergents. For example, if untreated enzyme preparations are exposed to phospholipase A for only brief periods there is a biphasic effect. This is shown by an initial increase in specific activity and drug binding which is followed by a reduction in both these parameters to near control levels after about 10 min of exposure. This can be seen in Fig. 3 where treatment with 20 units of phospholipase A was followed for 40 min. Under these conditions it is clear that the action of phospholipase A does not result in a marked reduction in the amount of [<sup>3</sup>H]ouabain bound to (Na<sup>+</sup> + K<sup>+</sup>)-ATPase.

On the other hand, if the enzyme preparations were first extracted with deoxycholic acid at 4°C, the biphasic effect of phospholipase A was lost. This is shown in Fig. 4 where it can be seen that while the initial levels of both ouabain-sensitive (Na<sup>+</sup> + K<sup>+</sup>)-ATPase activity and [<sup>3</sup>H]ouabain binding are

TABLE IV

COMPARISON OF THE SPECIFIC ACTIVITY AND EQUILIBRIUM LEVELS OF [<sup>3</sup>H]OUABAIN BOUND TO (Na<sup>+</sup> + K<sup>+</sup>)-ATPase AFTER VARIOUS TREATMENTS WITH DETERGENTS

Specific activity given as μmol ATP hydrolysis/mg protein per h at 37°C. Equilibrium level given as pmol [<sup>3</sup>H]ouabain bound/mg protein.

Treatment	n	Specific activity (mean ± S.E.)	Equilibrium level (mean ± S.E.)
None	14	15.2 ± 2.5	90.5 ± 6.1
Deoxycholic acid at 4°C	16	31.6 ± 3.2	146.5 ± 12.7
Deoxycholic acid + ATP at 30°C	3	96.9 ± 18.0	342 ± 55
Deoxycholic acid + ATP + Mg <sup>2+</sup> + Na <sup>+</sup> at 30°C	7	60.9 ± 4.8	295 ± 26
Sodium dodecyl sulfate + ATP at 30°C	3	133 ± 17	457 ± 51



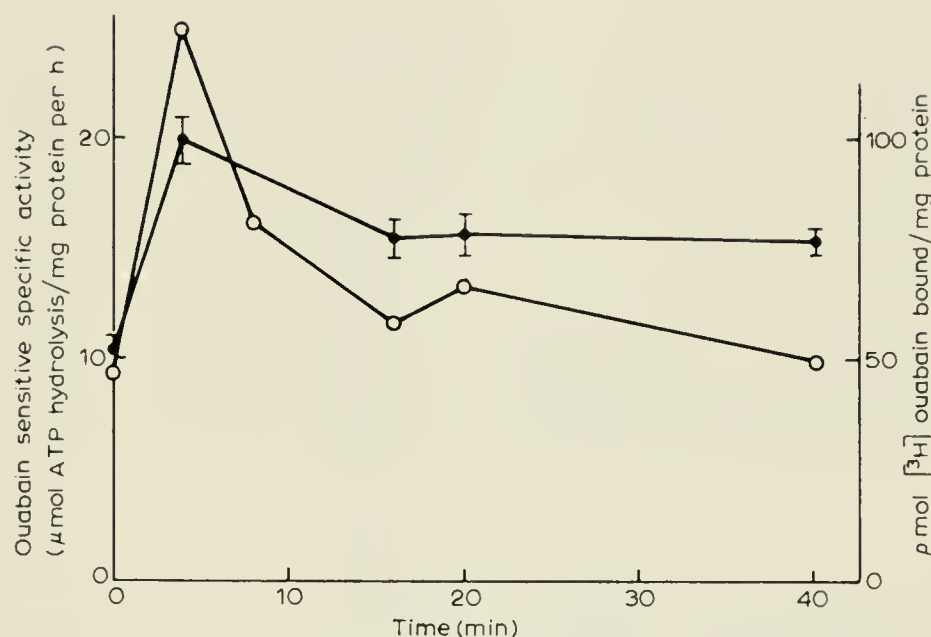


Fig. 3. Effect of progressive treatment at 37°C with 20 units of bee venom phospholipase A/mg protein on the specific activity and binding of [<sup>3</sup>H]ouabain at equilibrium to otherwise untreated membrane preparations of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase. ●, [<sup>3</sup>H]ouabain binding given as pmol/mg protein, values are the means ± S.E. of triplicate determinations from 1-ml aliquots taken after 5 min incubation at 37°C; ○, specific activity given as μmol P<sub>i</sub>/mg protein per h at 37°C; values are means of duplicate assays. 1 unit of phospholipase A hydrolyses 1 μmol of L-α-lecithin to lysolecithin and fatty acid per min at pH 8.5 at 37°C.

much higher than those of untreated enzyme preparations, the action of phospholipase A is to produce an immediate and progressive decline in the specific activity of the preparations which was accompanied by a fall in the amount of [<sup>3</sup>H]ouabain bound to these preparations under equilibrium conditions. However, under these conditions the amount of [<sup>3</sup>H]ouabain bound to the prepa-

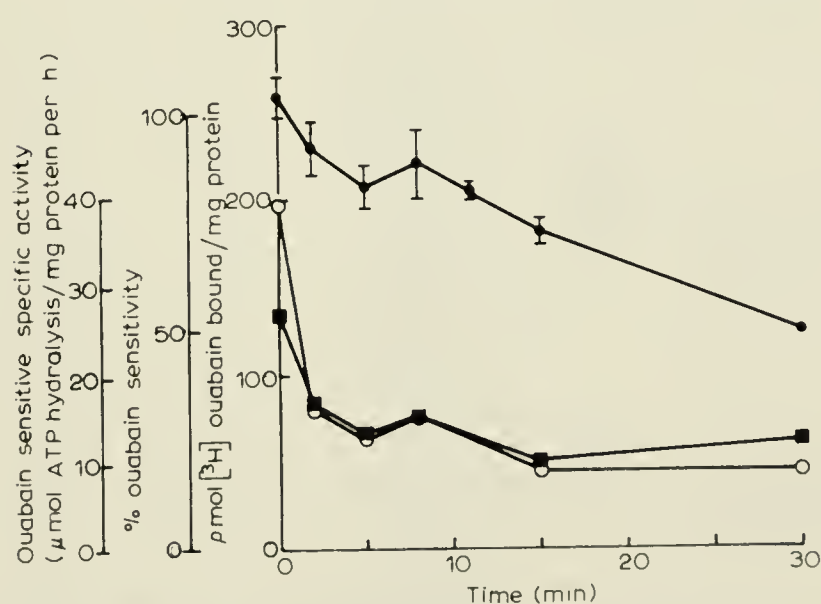


Fig. 4. Effect of progressive treatment at 37°C with 5 units of bee venom phospholipase A/mg enzyme protein on the specific activity and binding of [<sup>3</sup>H]ouabain at equilibrium to membrane preparations of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase after 10 min extraction with 0.1% deoxycholic acid at 4°C. ●, [<sup>3</sup>H]ouabain binding gives as pmol/mg protein, values are means ± S.E. of four times 1-ml aliquots taken after 5 min incubation at 37°C; ○, specific activity given as μmol P<sub>i</sub>/mg protein per h at 37°C; values are means of duplicate assays; ■, ouabain sensitivity expressed as percent of the total ATPase activity of the preparation assayed in duplicate ± 0.4 mM ouabain in the medium as described under Materials and Methods. Note the 4-fold reduction in concentration of phospholipase A from the experiments described in Fig. 3.



TABLE V  
EFFECT OF PHOSPHOLIPASE A ON SPECIFIC ACTIVITY, RATE AND EQUILIBRIUM BINDING OF [<sup>3</sup>H]OUABAIN TO (Na<sup>+</sup> + K<sup>+</sup>)-ATPase  
Specific activity given as μmol ATP hydrolysis/mg protein per h at 37° C. Rate [<sup>3</sup>H]ouabain binding given as pmol [<sup>3</sup>H]ouabain bound/mg protein per s at 37° C.  
Equilibrium level [<sup>3</sup>H]ouabain binding given as pmol [<sup>3</sup>H]ouabain bound/mg protein.

Experiment	Control			Phospholipase A		
	Specific Activity	Rate [ <sup>3</sup> H]ouabain binding	Equilibrium level [ <sup>3</sup> H]ouabain binding	Specific activity	Rate [ <sup>3</sup> H]ouabain binding	Equilibrium level [ <sup>3</sup> H]ouabain binding
a	38.7	3.40	194	5.4	2.57	157
b	44.4	3.80	216	8.3	2.28	155
c	29.4	3.34	192	4.6	1.97	133
d	19.6	1.92	102	2.4	0.80	70.7
e	17.6	2.25	102	2.09	0.92	77.4
f	15.8	2.13	79.1	2.05	0.83	57.3
g	18.9	2.07	74.0	2.12	0.59	50.6
Mean ± S.E.	26.3 ± 4.3	2.70 ± 0.29	137 ± 23	3.85 ± 0.90	1.42 ± 0.31	100 ± 17.6
Inhibition (%)	Nil	Nil	Nil	86.2 ± 1.0	50.8 ± 6.1	27.3 ± 1.7





ration does not fall in parallel with the reduction of enzyme activity. This is shown by the data from seven experiments given in Table V, where the reduction in specific activity which occurs after 5 min incubation with 5 units of phospholipase A is compared to the rate and amount of [ $^3\text{H}$ ]ouabain binding. Under these conditions the activity of the enzyme has been reduced to 14% of the mean control values, the mean rate of [ $^3\text{H}$ ]ouabain binding is 49% of the controls while the mean amount of [ $^3\text{H}$ ]ouabain which can be bound at equilibrium is only reduced to 73% of the control level. That is both the biochemical activity of the enzyme and the function of the binding sites are more susceptible to lipolysis with phospholipase A than are the number of sites available.

## Discussion

Although there are many reports in the literature concerning the binding of cardiac glycosides to particulate preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ , much of the data has been conflicting [24–27,31–39]. One contributing factor to this conflict has been the variable methodology employed by the numerous investigators. The studies of Erdmann and Schoner [10,11] have confirmed that under conditions of optimal phosphorylation of the enzyme viz. the presence of ATP,  $\text{Mg}^{2+}$  and  $\text{Na}^+$ , binding experiments conducted at equilibrium reflect the number of available cardiac glycoside binding sites per unit mass of enzyme, while very short term rate studies reflect the affinity of these sites rather than the number of sites available. When these distinctions are borne in mind some of the apparent differences reported in the literature can be resolved.

In recent years several laboratories have demonstrated that treatment of membrane preparations of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  with lipases destroys the thermal transitions which can be observed in the hydrolysis of ATP by this enzyme [12, 13,33,40–42], as well as reduces the rate of [ $^3\text{H}$ ]ouabain binding to the preparations in the presence of various combinations of ligands [32,36].

In the present work we have shown that the temperature dependence of the rate of [ $^3\text{H}$ ]ouabain binding to untreated enzyme preparations also displays a non-linear relationship with a break in Arrhenius plots at about  $25^\circ\text{C}$ . However, the differences in activation energy for this process which can be calculated above and below the critical temperature are somewhat less than the differences in the activation energies for ATP hydrolysis which were determined in paired experiments.

Our conclusions concerning the non-linear effect of temperature upon the rate of [ $^3\text{H}$ ]ouabain binding do not agree with those of Siegel and Josephson [23] or Schwartz and his colleagues [35,43]. The experiments by Siegel and Josephson [23] were conducted after 15 min of incubation which under our conditions, would neither reflect the initial rates nor the equilibrium levels of drug binding, particularly in the lower temperature range examined.

Conversely, the experiments reported by Schwartz and his colleagues [35,43] should provide data reflecting the affinity of cardiac glycoside binding sites similar to that observed in our experiments. Although in the experiments of Wallick and Schwartz [43] there are an insufficient number of data points for the construction of Arrhenius plots suitable for the Bogart [22] analysis



we employ, inspection of their data reveals that their results might also be described as non-linear. Recalculation of their data suggests that like our own findings, the differences in activation energies for [ $^3\text{H}$ ]ouabain binding above and below the critical temperature are not as marked as is the case with ATP hydrolysis by these membrane enzyme preparations. Presumably, both our findings and those of Wallick and Schwartz [43] indicate that the binding of [ $^3\text{H}$ ]ouabain to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  is less influenced by the physical state of the membrane lipids than is activation of the system by cations [12,13,44].

However, it is apparent that lipids do play at least some part in the temperature dependence of [ $^3\text{H}$ ]ouabain binding to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  as detergent treatment of the preparations resulted in the loss of the discontinuity in temperature dependence and the observation of linear Arrhenius plots. The comparative ease with which detergents remove this effect suggests that the lipids which influence [ $^3\text{H}$ ]ouabain binding are not as closely associated with the membrane protein as are those which are responsible for modulation of the temperature dependence of ATP hydrolysis [12,13]. Perhaps the former is a so-called "bulk" lipid phenomenon while the latter is more likely to be the immobilized boundary lipid type recently described by Metcalfe and his colleagues [45,46] for  $\text{Ca}^{2+}\text{-ATPase}$ . In the limited number of experiments we attempted here, it is of considerable interest that although reconstitution of phospholipase A-treated preparations with phosphatidylserine is able to regain the characteristic non-linear temperature dependence for ATP hydrolysis that we have reported before [12,13], that is not necessarily the case for [ $^3\text{H}$ ]ouabain binding. As only one set of experimental conditions for reconstitution with phosphatidylserine was employed here (3 mg phosphatidylserine/mg protein at  $37^\circ\text{C}$  for 10 min) it is clear that these attempts at reconstitution will have to be extended before the role of phosphatidylserine in [ $^3\text{H}$ ]ouabain binding to  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  can be decided.

Nevertheless, the partial dependence of [ $^3\text{H}$ ]ouabain binding upon lipids is demonstrated by the effects of phospholipase A treatment upon the amount of [ $^3\text{H}$ ]ouabain bound to the enzyme preparation at equilibrium at  $37^\circ\text{C}$ . With untreated enzyme preparations there is an initial increase in the amount of drug binding which falls off after about 5 min of incubation, but does not fall below control levels during further treatment with phospholipase A. In these experiments the pattern of specific activity of ouabain-sensitive  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  activity parallels the changes in [ $^3\text{H}$ ]ouabain binding which were observed.

On the other hand, if the membrane enzyme preparations are first treated with the detergents deoxycholic acid or sodium dodecyl sulfate at  $30^\circ\text{C}$  (in the presence of ATP) there is a very marked increase in both the specific activity of ouabain-sensitive  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  and the amount of [ $^3\text{H}$ ]ouabain bound to the enzyme. This increase in [ $^3\text{H}$ ]ouabain binding is now very sensitive to treatment with phospholipase A and is reduced by about 50% in 15 min incubation with a much reduced concentration of lipase.

It should be noted that the final levels of ouabain-sensitive ATPase activity and [ $^3\text{H}$ ]ouabain binding which are reached after treatment with phospholipase A are very similar whether detergent-treated or untreated control enzyme was used; apparently this residual enzyme activity and its associated [ $^3\text{H}$ ]ouabain binding sites are very resistant to attack by this lipase. In agreement with the





earlier work of Taniguchi and Iida [36], and the recent conclusions of Hansen [47], which were published while this manuscript was in preparation, we also conclude that there are two different types of [ $^3\text{H}$ ]ouabain binding sites available in membrane preparations of ( $\text{Na}^+ + \text{K}^+$ )-ATPase. Because of the marked sensitivity of only one of these sites to lipolysis with phospholipase A following detergent extraction, it seems very likely that not only are these sites located in different regions of the membrane but that only one site is closely associated with a phospholipid component of the membrane. Whether these sites are equally accessible for cardiac glycoside binding in situ, or whether either of these sites represents a more pharmacologically active "receptor" cannot be determined from the present work, but it is possible that variations in membrane lipids will exert differential effects on the drug-receptor interactions at these different sites. Whether either of these sites resemble the clinically important cardiac glycoside receptor of cardiac muscle remains uncertain [48–50].

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## APPENDIX VII



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## AN ELECTRON SPIN PROBE STUDY OF (Na<sup>+</sup> + K<sup>+</sup>)-ATPase-CONTAINING MEMBRANES

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### Summary

The modulating effect of membrane lipids on enzyme function has been described by several investigators. We have used the spin probe *N*-oxyl-4',4'-dimethyloxazolidine-12-keto methyl stearate (M 12-NSE) to study this interaction in ox brain membranes enriched with (Na<sup>+</sup> + K<sup>+</sup>)-ATPase. This methyl ester of stearic acid is practically insoluble in aqueous media, and consequently spectra of M 12-NSE-labelled preparations are free of "liquid lines".

At least two types of spectra may be obtained when ox brain microsomes are spin labelled with M 12-NSE, indicating the presence of two distinct binding sites. At one site the spin label is relatively unrestricted and gives rise to an isotropic spectrum. A second spectrum, which is obtained from spin label at another site, is similar to that which is observed after incorporation of M 12-NSE into phospholipid bilayers. This suggests that this latter site is within the core of the microsomal membrane.

The two binding sites differ in their affinity for the spin probe. The low affinity site is both more abundant in crude preparations and is more easily removed by detergent treatment; spin labels at this site produce isotropic spectra. The high affinity sites are fewer in number and produce broad spectra. In addition these high affinity sites increase in concentration as the enzyme undergoes purification.

The two sites are quite distinct in their sensitivity to ascorbic acid, the low affinity site showing a considerably greater rate of reduction by this agent.

This study also demonstrates that the delipidation effects of sodium dodecyl sulfate and sodium deoxycholate on (Na<sup>+</sup> + K<sup>+</sup>)-ATPase-enriched microsomes from ox brain are not identical.

It is suggested that the two spin probe binding sites represent two different lipid domains, one of which is very closely associated with the (Na<sup>+</sup> + K<sup>+</sup>)-

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Abbreviations: ESR, electron spin resonance spectroscopy; M 12-NSE, *N*-oxyl-4',4'-dimethyloxazolidine-12-keto methyl stearate; 5-NS, *N*-oxyl-4',4'-dimethyloxazolidine-5-keto stearic acid.





ATPase enzyme and may reflect a protein-directed phospholipid specificity for this enzyme.

## Introduction

The preparation of ( $\text{Na}^+ + \text{K}^+$ )-ATPase containing microsomes by differential centrifugation with or without sodium deoxycholate extraction, can result in products that differ significantly in the specific activity of the enzyme. However, the temperature sensitivity of the enzyme is not altered [1]. In contrast, the binding of ouabain to this enzyme, which is also temperature sensitive, is markedly different after extraction of the microsomal preparations with sodium deoxycholate or other detergents [2]. This temperature sensitivity is best described by the non-linearity found in Arrhenius plots which are believed to arise from phase transitions or phase separations of the membrane lipids [3]. Whether these transitions pertain to a specific lipid or a number of lipids is not clear. The use of biophysical techniques such as fluorescence spectroscopy and ESR to investigate the physical behaviour of lipids is extensively used, and we are currently engaged in an electron spin probe study of microsomal membranes rich in ( $\text{Na}^+ + \text{K}^+$ )-ATPase. A number of review articles of this object are now available [4–6]. It is widely believed that both steroid and fatty acid spin probes intercalate in the membrane bilayer [6] and are consequently used to detect physical changes in the characteristics of the lipid matrix. However, it is not yet possible to precisely locate the spin label which may report from specific domains, from the bulk lipid, or from both [7,8]. In addition, the spin probes may also dissolve in the aqueous phase and give rise to “liquid lines” [9, 10]. Spin probes in aqueous solution are rapidly reduced by ascorbic acid and a study of the rate of this reaction can provide information as to the location of the spin probe in the bilayer [11–13]. In the present study we present evidence to show that the spin probe, the *N*-oxyl-4',4'-dimethyloxazolidine-12-keto derivative of methyl stearate (M 12-NSE) which is essentially insoluble in aqueous media, will bind non-covalently at more than one site to ox brain microsomes rich in ( $\text{Na}^+ + \text{K}^+$ )-ATPase. Preliminary communications of this work have been made at recent symposia [27,28].

## Materials and Methods

*Microsomes and liposomes.* The preparation, delipidation procedures, and biochemical assay of the ( $\text{Na}^+ + \text{K}^+$ )-ATPase of the ox brain microsomes have been described recently by Charnock et al. [2].

Liposomes were made from dimyristoyl phosphatidylcholine (Serdary Research Laboratories, Ontario) or from the lipids extracted from ox brain microsomes. Weighed amounts of lipid were added to a buffered sucrose solution (0.25 M sucrose, 5 mM  $\text{Na}_2\text{EDTA}$ , pH 7.6, with Tris) and the mixtures sonicated in a Cole-Parmer Ultrasonic Cleaner, Model 884S-4, until a clear solution was obtained.

*Electron spin resonance.* The spin label, a methyl ester of stearic acid with the nitroxyl on the 12-C position M (12-NSE), was a gift from Dr. J.K. Raison,



Macquarie University, Sydney, Australia. A methanol solution of M 12-NSE was evaporated in a stream of nitrogen and the suspension of microsomes or liposomes was added to the label. After incubation for 10 min at 37°C, the sample was stored at 0°C until it was used for spectral investigation. There was no difference in the spectra that were obtained whether the labelling procedure was carried out directly in the glass capillary sample tube (1.1 mm internal diameter) or in a separate vessel and subsequently transferred. No sample was stored for longer than 24 h.

The ESR spectra were obtained using a Varian V-4502 EST spectrometer. This instrument is equipped with an Alpha Model 3039 digital NMR gaussmeter for magnetic field calibration and a Varian-4557 temperature controller. The temperature of the sample chamber was examined by a copper-constantin thermocouple, to 0.1°C. Microwave frequencies were monitored with a HPX 532B frequency meter.

A number of methods are currently available for the quantitation and semi-quantitation of ESR spectra. Using some approximations, McConnell [14], Kivelson [15] and Freed and Fraenkel [16] have developed mathematical methods for the calculation of rotational correlation times of spin probes incorporated into rigid matrices. These approaches are only applicable to rapid motion, such as that seen as isotropic spectra from spin probes in solution. The ESR spectra of fatty acid spin probes in lipid bilayers are often indicative of anisotropic motion [17,18] and a measure of the membrane fluidity, the order parameter, can be obtained for spectra such as these [17,18]. Other methods that have also been used for broad spectra, make use of one or more of the peak heights and although they are semiquantitative, these simple approaches can be very useful in the interpretation of ESR spectra obtained from spin-labelled biomembranes. In this report we have generally confined our results to a qualitative description of the different spectra; where quantitative data is presented, peak heights are used as described in the legends.

The reduction of M 12-NSE by ascorbic acid was examined by exposing the labelled material to 2 mM ascorbic acid, freshly prepared, (pH 7.5 with Tris) and monitoring the changes in peak heights with time.

## Results

In the application of electron spin resonance spectroscopy to the examination of biomembranes, two complications are commonly encountered. One of these is the appearance of "liquid lines" due to spin probe that has dissolved in the aqueous phase [9,10], and a second problem is the possibility of line broadening due to interactions between the electrons of the spin probe molecules when pooling or clustering occurs [19,20]. Both these effects can be avoided however, by reducing the amount of spin probe that is employed, but some limitations must first be defined. Preliminary experiments [27,28] demonstrated that any spectral contribution from the empty capillary tube, the residual spin label on the walls of the sample tube, or from label dissolved in the buffered sucrose medium were all negligible and could be contained by limiting the gain settings to a narrow range. In particular we established that at the probe concentration used in the experiments reported here, even after 24 h exposure





to the aqueous buffer, a negligible amount of dissolved label could be detected.

In these preliminary experiments we also examined the spectra of M 12-NSE in liposomes (smectic mesophases). Liposomes made from extracted membrane lipids or from dimyristoyl phosphatidylcholine, when spin labelled by the method we have described, showed a rapid incorporation of spin probe as is commonly reported [21,22]. The spectrum of labelled liposomes displayed the characteristic line shape of a spin probe oriented perpendicular to the plane of the bilayer [17,18]. Moreover, varying the probe : phospholipid ratio from 1 : 14 to 1 : 110 did not cause any major change in the line shape of the spectrum.

#### *Spectral variations of spin-labelled microsomes*

Recent work in our laboratory has shown that three characteristics of ( $\text{Na}^+ + \text{K}^+$ )-ATPase from ox brain microsomes, namely the specific activity, the rate of ouabain binding, and the effects of temperature on these enzyme characteristics are not equivalent in untreated and detergent-extracted preparations [1, 2]. Our present experiments also indicate that when untreated or detergent-extracted microsomal preparations of ( $\text{Na}^+ + \text{K}^+$ )-ATPase are spin labelled with M 12-NSE, the differences in the spectra which are obtained reflect the variations in the pretreatment of the membranes. Fig. 1 shows a representative sample of the different types of spectra obtained from a variety of enzyme preparations.

Similar to our findings with dimyristoyl phosphatidylcholine liposomes, in the presence of a suspension of ox brain microsomes, M 12-NSE will diffuse rapidly from the glass surface into the microsomal membrane. The shortest

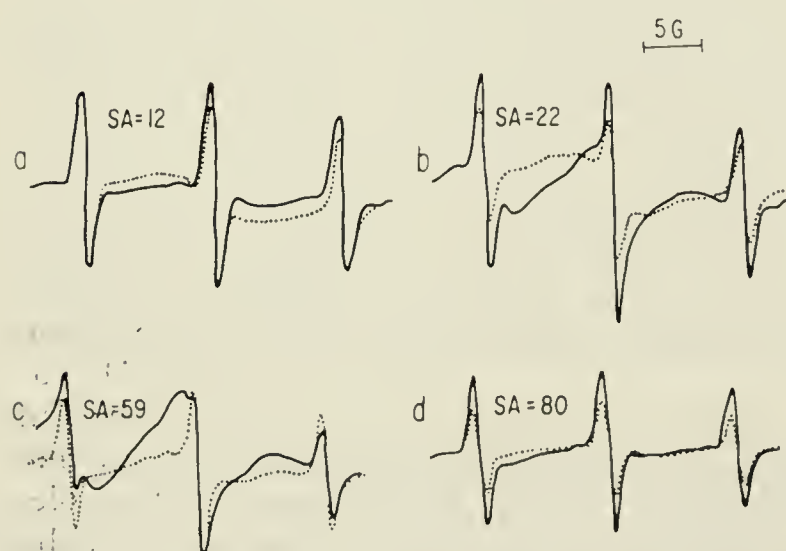


Fig. 1. M 12-NSE in ox brain microsomes. Untreated ox brain microsomes (a,b,c) and after 2 min extraction with 0.1% sodium deoxycholate in the presence of 2 mM ATP and 80 mM NaCl (d) were labelled in sample tubes containing 2 nmol M 12-NSE. Diffusion of the probe was allowed to proceed for at least 1 h prior to spectral examination. Two membrane concentrations from each of the four enzyme preparations were examined. The specific activities (S.A.) of the preparations were, respectively 12, 22, 59 and 80  $\mu\text{mol P}_i/\text{mg protein per h}$  as shown on the figure. The membrane concentrations in  $\mu\text{g protein/sample}$  were as follows, in each set of data the lower concentration is given as the broken line: a, 1.25 (.....) and 2.5 (—); b, 4.0 (.....) and 16.0 (—); c, 6.6 (.....) and 21.0 (—); d, 2.6 (.....) and 25.5 (—).





exposure time that was examined was 2 min at 37°C, after which time no further diffusion occurred. In Fig. 1a, a 2-fold difference in membrane concentration is compared. In these experiments both spectra have three sharp peaks equally spaced and of similar intensity, not unlike the isotropic spectrum of a rapidly tumbling nitroxide label in a solvent of low viscosity [6]. This type of spectrum, for which rotational correlation times can readily be determined [14–16], is characteristic of relatively unrestricted labels in the membrane. The lines in the two spectra have very similar intensities, but the low membrane concentration has some distortion of the base line due to residual M 12-NSE on the surface of the sample tube. In Fig. 1b, a 4-fold difference in concentration of membrane protein is compared. Here again the two spectra are similar in appearance and resemble the isotropic spectrum described above. There is, however, a difference in the two spectra. A broad absorption, particularly between the low-field and mid-field peaks, is apparent in the spectrum at the higher membrane concentration. This phenomenon is only observed at the lower probe : membrane ratio ( $1.2 \cdot 10^{-4}$   $\mu\text{mol}$  M 12-NSE : 1  $\mu\text{g}$  protein) which suggests that the increase in membrane concentration (16  $\mu\text{g}$  protein/sample) is responsible for the alterations in spectral line shape.

Fig. 1c shows a pair of spectra obtained with a 3-fold difference in membrane concentration. Although this concentration difference is intermediate to the two previous pairs of spectra, the divergence in spectral line shape is by far the greatest. At the lower membrane concentration (6.6  $\mu\text{g}$  protein) the spectrum is typically isotropic, but when the membrane concentration in the sample is raised to 21.3  $\mu\text{g}$  protein the spectrum is no longer isotropic. The form of this spectrum suggests the presence of two probe-binding sites, one of which is highly mobile (isotropic spectrum) and one where the labels are restricted (broad spectrum). Finally, in Fig. 1d we show a pair of spectra that were obtained by spin-labelling microsomes that had been extracted with sodium deoxycholate but in the presence of sodium and ATP [2]. The line shapes of both spectra are typically isotropic despite the fact that the membrane concentration of the two samples differs by an order of magnitude.

It is clear that the observed spectral variations are markedly influenced by membrane concentration. However, if the first three pairs of spectra in Fig. 1 are examined collectively, it is apparent that in addition to the membrane concentration, the specific activity of the enzyme preparations also influences the spectrum. In addition, the spectra shown in Fig. 1d indicate that treatment of the microsomes with detergents also produces changes in the spectral shape.

#### *The effect of membrane (protein) concentration on the spectrum*

To further characterize the effects of membrane concentration on spectral type we have examined the spectra of samples containing a fixed amount of probe (2 nmol) and varying amounts of membrane from a single enzyme preparation. A selection of these spectra is shown in Fig. 2. Although the spectra again vary with the membrane concentration, they can be grouped into three categories. At the low membrane concentrations the spectrum is a typical isotropic spectrum characteristic of unrestricted labels. At the very high membrane concentrations, the spectrum is considerably broadened and now resembles the line shape of the spin-labelled dimyristoyl phosphatidylcholine lip-



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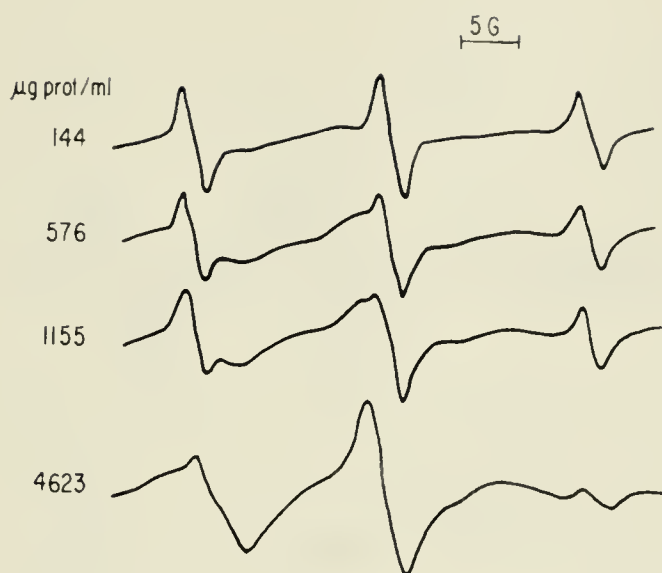


Fig. 2. The effects of membrane concentrations ( $\mu\text{g}$  protein) on the spectrum of microsomes labelled with M 12-NSE. Increasing concentrations of untreated ox brain microsomes were added to sample tubes each containing 2 nmol of M 12-NSE. Diffusion of the probe was allowed to proceed as described for Fig. 1. Note the change in spectrum from sharp to broad as the concentration of the membrane increases.

somes. At intermediate membrane concentrations, the shape of the spectrum is a superposition of the spectra at the extremes, with the relative intensities being determined by the membrane concentration. Thus at low membrane concentrations, the probe binds to sites that permit greater motional freedom, whereas at high membrane concentrations a site that restricts the motion of the nitroxyl is preferred. We define these two spectra as “unrestricted” and “restricted”, respectively. The exact ratio of probe : membrane at which a restricted spectrum is obtained is constant for any one microsomal preparation, but may vary from one preparation to another. This latter variation bears some correlation to the degree of purification and specific activity of the enzyme and further evidence for this claim is provided later.

Spectra which are intermediate to the two limiting types described above, are a separate group constituting a mixture of two spectral types. They show the three peaks which are characteristic of the three sharp line spectrum and also display evidence of intermediate peaks. At present these mixed spectra cannot be characterized further. Presumably they arise if both restricted and unrestricted labels contribute to the spectrum.

The effect of 2 mM ascorbic acid on the two sites was also examined. For the unrestricted labels, 50% reduction in peak height intensity had occurred within the first 10 min. This is twice as long as the rate observed for the reduction of M 12-NSE when present as a solution in methanol. In contrast, after 90 min the peak height intensity for restricted spectra had decreased by less than 10%. This differential reduction by ascorbic acid has been reported by others and is believed to reflect the availability of the nitroxyl group for reduction [11–13].

#### *The effect of enzyme purification on the spectrum*

The ESR data described above strongly suggest a correlation between enzyme purity and the ESR spectrum. Furthermore, we have previously shown





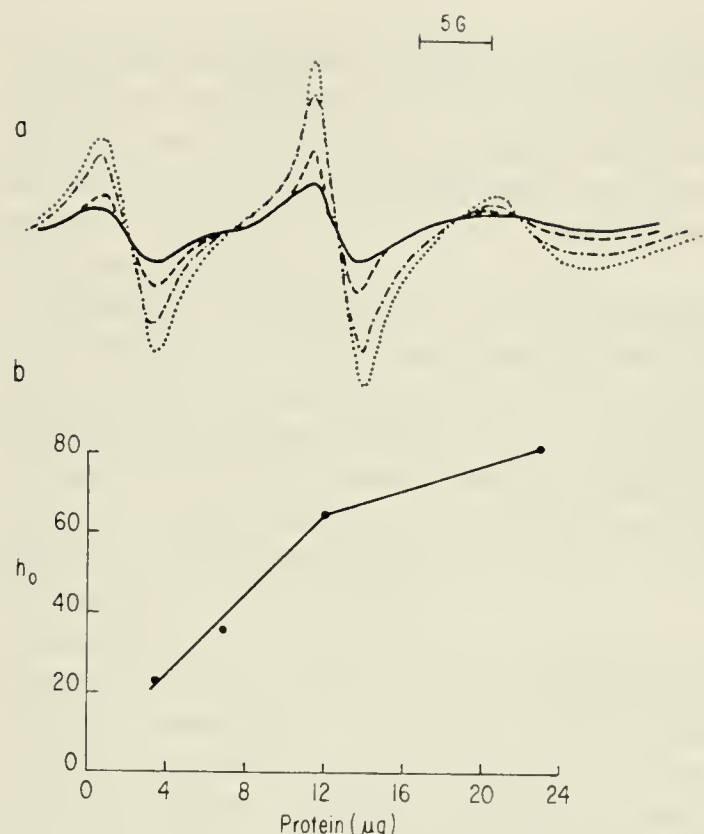


Fig. 3. The effects of membrane concentration after enzyme purification. Increasing concentrations of sodium dodecyl sulfate-extracted ox brain microsomes were added to sample tubes each containing 2 nmol M 12-NSE. Diffusion of the probe was allowed to proceed as described for Fig. 1. (a) Spectral comparison of samples containing 22.8 (· · · · ·), 11.9 (· — · — ·), 6.9 (— — — —) and 3.4 (——) µg protein per sample tube. (b) Plot of mid-field peak height ( $h_0$ ) vs. protein concentration.

that ox brain microsomes when exposed to low concentrations of sodium dodecyl sulfate in the presence of ATP will undergo dramatic increases in ( $\text{Na}^+ + \text{K}^+$ )-ATPase activity [2]. Thus we have examined the spectra of sodium dodecyl sulfate-treated enzyme preparations. Fig. 3a shows the results obtained from a more active preparation (158 µmol  $\text{P}_i$ /mg protein per h) than those found after treatment with sodium deoxycholate. Four different membrane concentrations were labelled with a constant amount of 2 nmol of M 12-NSE. Although the amount of membrane added was varied from 3.4 to 23 µg protein, it is quite clear that the spectra are all of the restricted type. This supports the notion that high activity enzyme preparations have a greater proportion of restricted sites than do untreated preparations of lower biochemical activity. If the peak height is plotted against membrane concentration, Fig. 3b, a linear plot is obtained for the first three points. This linearity is good evidence for homogeneous labelling in this system and also for the loss of unrestricted probe-binding sites in sodium dodecyl sulfate-treated microsomes. The highest membrane concentration shows a flattening of the curve, presumably because the amount of M 12-NSE probe is now the limiting factor. Three sodium dodecyl sulfate-treated preparations with elevated specific activities (158, 144 and 142 µmol  $\text{P}_i$ /mg per h) were spin labelled and examined. In all cases the restricted spectrum was observed with no evidence of mixed or unrestricted spectra.





## Discussion

We have described a method which permits the spin labelling of biological membranes by diffusion of the spin probe from the surface of the glass sample tube. By using M 12-NSE, "liquid lines" are avoided because the solubility of this label in an aqueous medium is very low. This method permits the use of minimal amounts of spin probe, and also avoids contamination of the sample with organic solvents.

Liposomes of dimyristoyl phosphatidylcholine or extracted membrane lipids, demonstrate the characteristic behaviour of a typical nitroxyl spin label intercalated into a lipid bilayer. The spectrum was typical of a nitroxide restricted to motion within a cone described by the methylene chain [23]. Saturation of the liposome with spin label did not alter the line shape of the spectrum. Thus the bilayers of liposomes made with dimyristoyl phosphatidylcholine or with lipid extracts of microsomes are homogeneous with respect to binding sites for M 12-NSE.

Conversely, when ox brain microsomes are spin labelled with M 12-NSE a variety of spectra may result. At high probe : membrane ratios the spectrum is isotropic, resembling that of a nitroxide tumbling freely in solution. Presumably such a spectrum must arise from nitroxides residing at unrestricted sites. At the low probe : membrane ratios the spectrum becomes broadened and now bears similarity to that of spin-labelled liposomes. In this situation the nitroxide is in a location that partially restricts its motion. These two limiting cases are quite distinct and are best interpreted as originating from spin probes at two independent sites, an unrestricted motion site, and a restricted motion site. The spin labelling of the two sites with M 12-NSE is clearly influenced by the probe : membrane ratio, and the evidence suggests that although the restricted sites are fewer in number they possess greater affinity for the M 12-NSE.

The effect of membrane concentration on the spectrum varies from one enzyme preparation to another, and this variation can be correlated with the degree of purification of the membrane-bound ( $\text{Na}^+ + \text{K}^+$ )-ATPase. As the enzyme is purified the activity increases and this results in a loss of unrestricted sites with a corresponding increase of restricted sites. However, it is possible to obtain a restricted spectrum from a preparation with low specific activity, if the probe : membrane ratio is greatly reduced. Presumably an unrestricted spectrum does have a weak underlying component from restricted sites.

The spectra of sodium deoxycholate-extracted microsomes are influenced by membrane concentration, and in this respect are qualitatively similar to those of untreated microsomes. Brief exposure to sodium deoxycholate in the presence of ATP and  $\text{Na}^+$  resulted in an elevation of the specific activity of the enzyme, apparently without a marked reduction in the number of unrestricted sites. By contrast, extraction with sodium dodecyl sulfate resulted in an increase in specific activity which was accompanied by a decrease in unrestricted sites. Presumably, under the conditions employed by us, this detergent interacts with proteins and also with lipids, as has been previously reported [24]. Preliminary examination shows a marked increase in lipid : protein ratios after extraction with sodium dodecyl sulfate compared to either untreated or sodium deoxycholate-extracted preparations.



The following summary characterizes the two sites that we have defined as unrestricted and restricted for the non-covalent binding of M 12-NSE to ox brain microsomes. (1) Two types of spectra may be obtained when ox brain microsomes are spin labelled with M 12-NSE; (2) These two spectral types are due to two different binding sites; (3) These two binding sites differ in their affinity for the spin probe; (4) The low affinity site is more abundant in crude preparations and is easily removed. The high affinity sites increase in concentration as the enzyme undergoes purification; (5) The two sites are quite distinct in their sensitivity to ascorbic acid, the low affinity site showing a considerably greater rate of reduction by this agent.

Since the reporter molecule M 12-NSE is a lipid probe, it is a reasonable assumption that the two sites reflect two different lipid domains. Although specific localization is not possible at present some comment is warranted. The domain of the unrestricted site, if it is in the core of the bilayer is a region of very great fluidity. Using the method of Kivelson [15], we have calculated tumbling times which range from  $1.4 \cdot 10^{-10}$  s for a typical isotropic spectrum to  $1.9 \cdot 10^{-10}$  s for an isotropic spectrum with distinct evidence of restricted labelling (i.e. presence of secondary peaks). These values are similar to the tumbling times we determined for the water-soluble stearic acid probe 5-NS in 0.25 M sucrose buffer;  $1.2 \cdot 10^{-10}$  s. A lipid domain in the core of a membrane with similar viscosity to 0.25 M sucrose does not seem likely. However, typical isotropic spectra such as those described in this work, have been described for the lipid core of a smectic bilayer in the liquid crystalline state [16]. An alternative location would be the membrane-water interface. Such a position would comply with the rapid reduction of the spin label by ascorbic acid, but would require a bending of the polymethylene chain as described by Cadenhead et al. [25].

An intramembrane domain for the restricted sites is a definite possibility because of their similarity to the probe binding sites of liposomes. The correlation between restricted sites and specific activity of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  implies a lipid specificity for the enzyme protein, similar to the "liquid clustering" reported by Lee et al. [26]. Presumably the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  macromolecule, when present in ox brain microsomes is capable of selective aggregation of a phospholipid cluster of its own preference, not unlike the lipid selectivity displayed by the  $(\text{Mg}^{2+} + \text{Ca}^{2+})\text{-ATPase}$  macromolecule from sarcoplasmic reticulum [29].

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